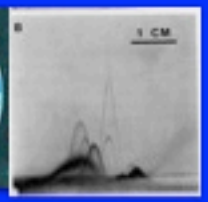
EUREKA - THE STANDARD MODEL OF P. AERUGINOSA INFECTION IN CYSTIC FIBROSIS



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Tanja Pressler

Søren Molin

Gerd Döring

Burkhard Tümmler

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Michael Givskov

Bill Costerton

Christian Buchwald

Peter Østrup Jensen

Lotte Mandsberg

Hengzhuang Wang

Baoleri Lee

Kasper Aanæs

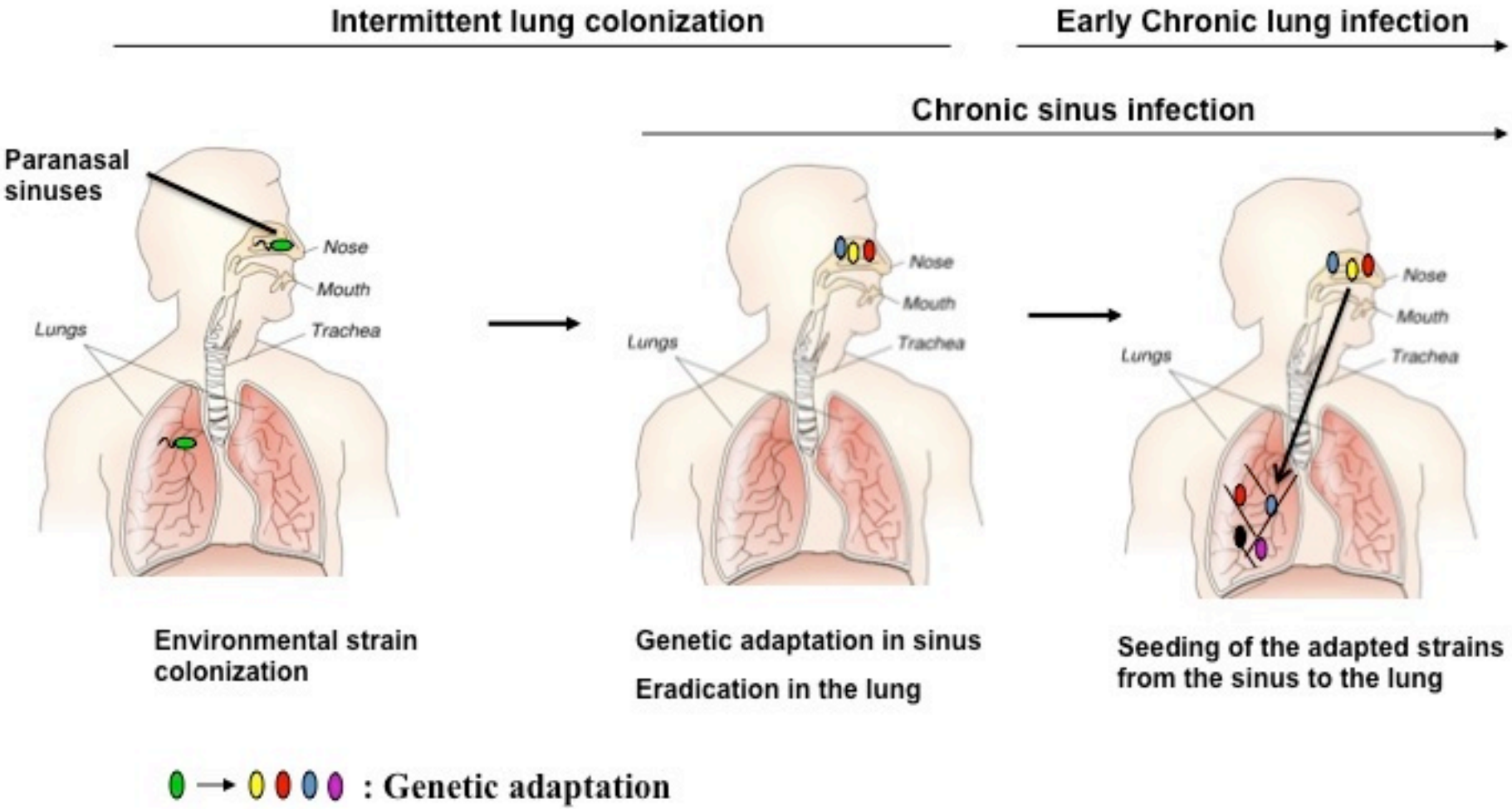
Mette Kolpen

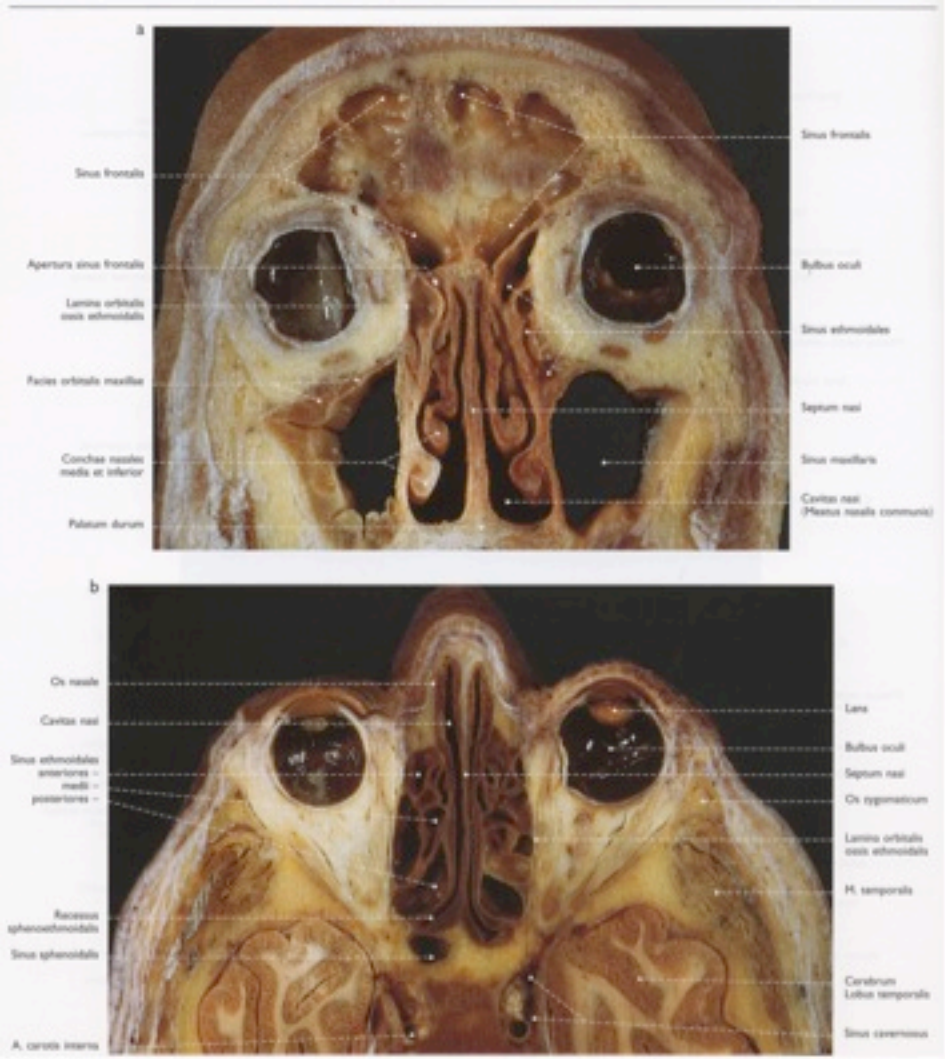
Christine R. Hansen

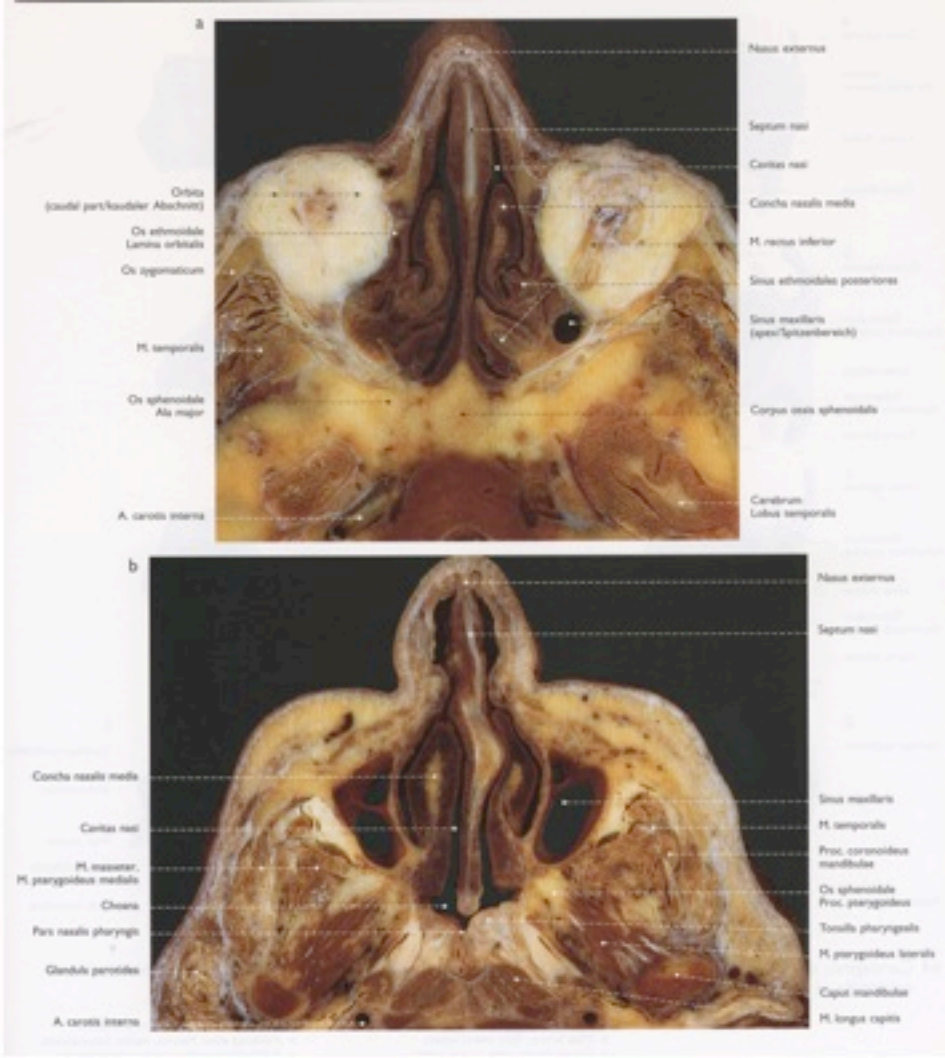
Christina Dalbøge

(Folkesson et al. Nature Reviews, submitted 2011)

Chronic P. aeruginosa biofilm lung infection in cystic fibrosis







Paranasal sinuses
(Köpf-Maier: Atlas of Human Anatomy. 5th ed. Vol. 2. Karger, Basel, 2000)

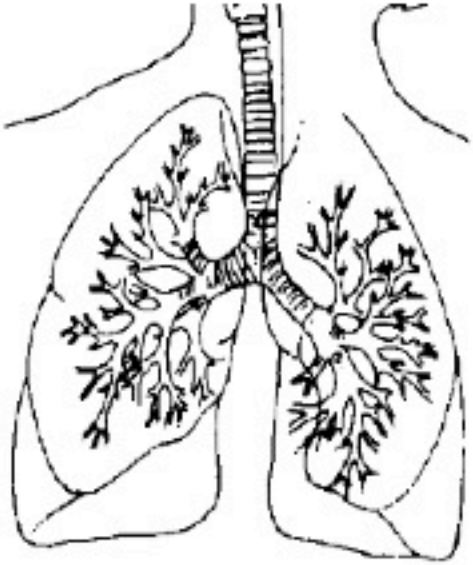
CAS FESS IN CF PATIENTS

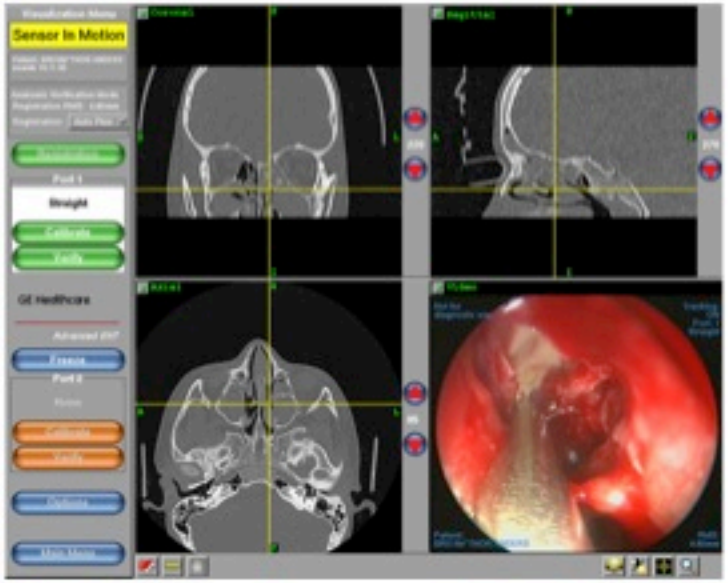
INTERMITTENT P. A. LUNG COLONIZATION:

Sinus vs lung (N): 22 = 91% same clone

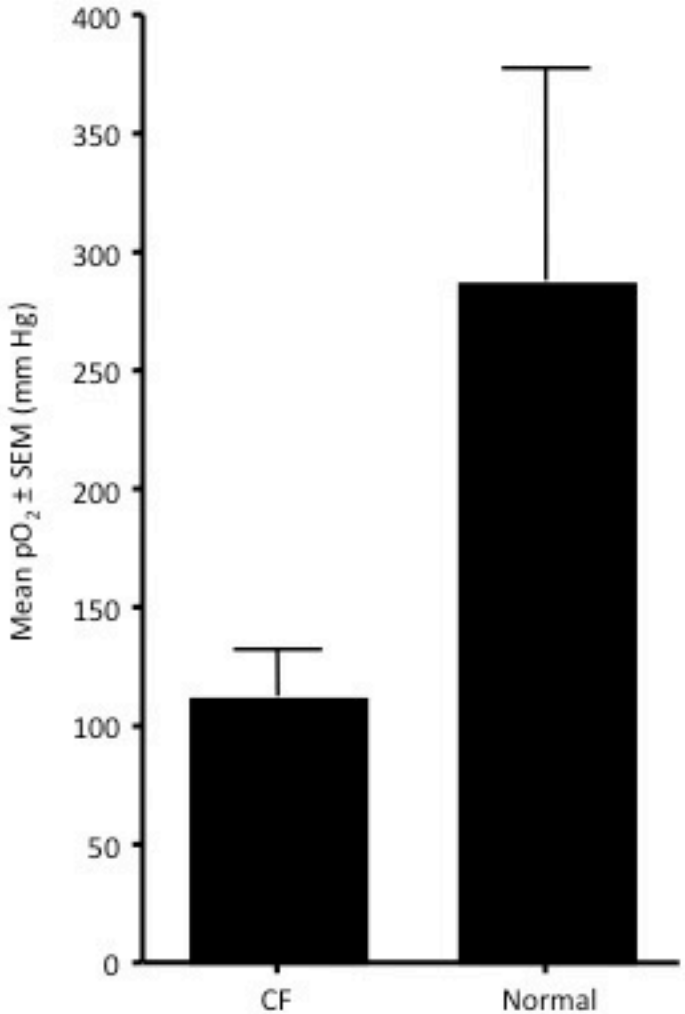
CHRONIC P.A. LUNG INFECTION:

Sinus vs lung (N): 18 = 100% same clone

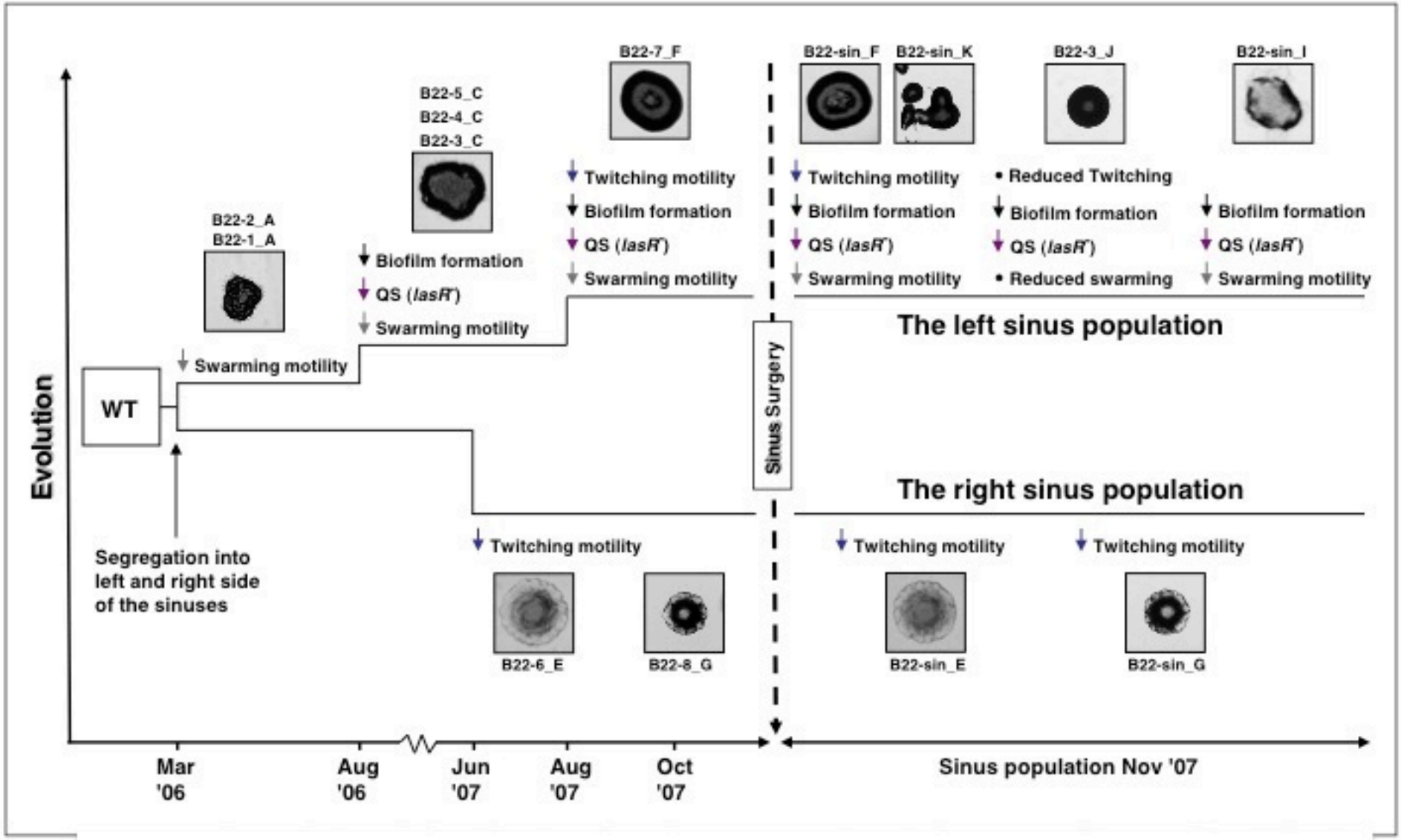




PO_2 in the mucosa of all maxillary sinuses (t-test), p<0.0263, (Aasnæs, K., Rickewlt, L.F., Johansen, H.K., von Buchwald, C., Pressler, T., Høiby, N., Jensen, P.Ø.: Decreased mucosal oxygen tension in the maxillary sinuses in patients with cystic fibrosis. J. Cystic Fibrosis, submitted 2010). J. Cystic Fibrosis, 2011, online: http://dx.doi.org/10.1016/j.cf.2010.12.002.



(Hansen, Rau, Johansen, Ciofu, Jelsbak, Yang, Folkesson, Jarmer, Aanæs, Buchwald, Høiby, Molin: Evolution and diversification of P. aeruginosa in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection. ISME Journal, online publ. doi: 10.1038/ismej.2011.83; 2011)



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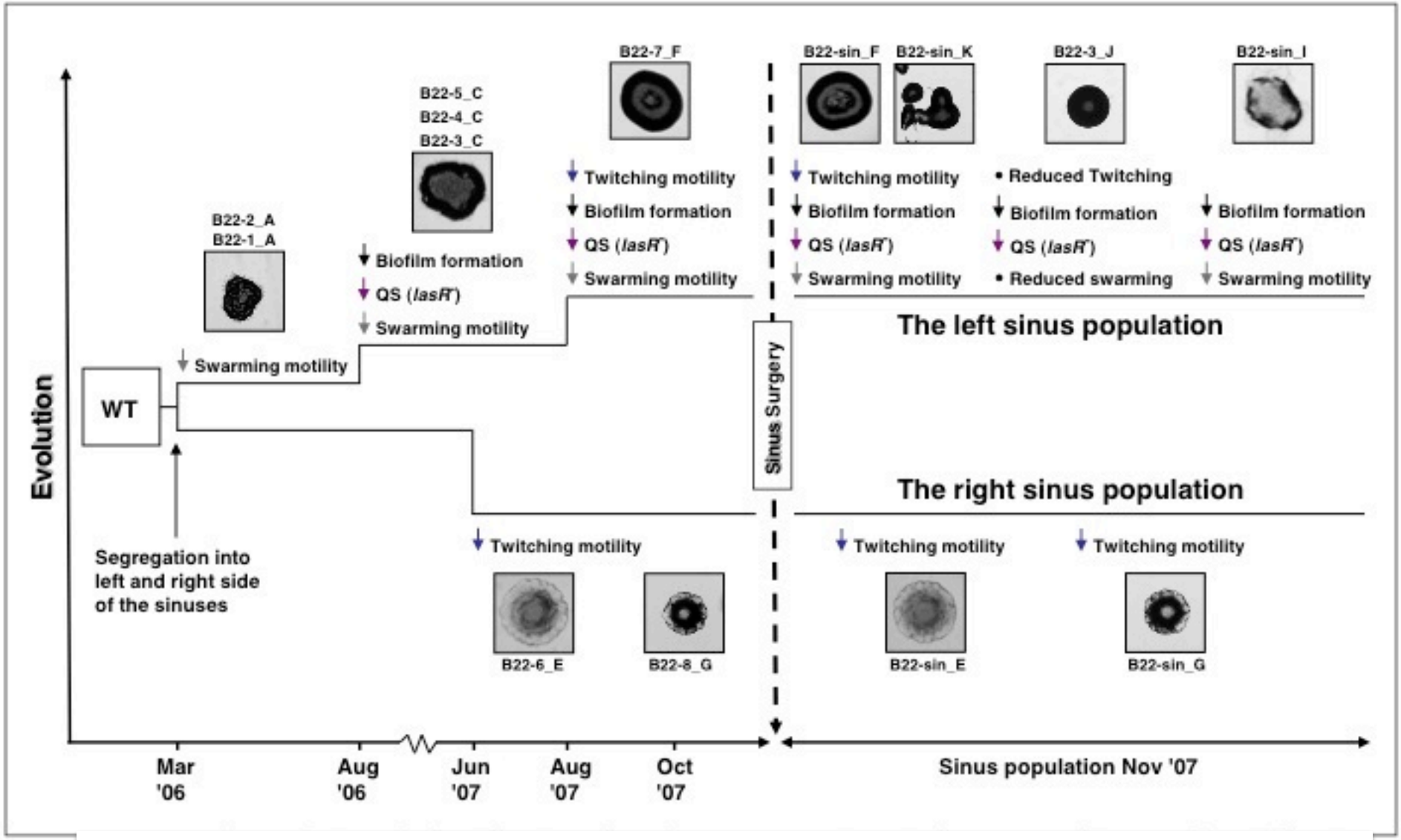
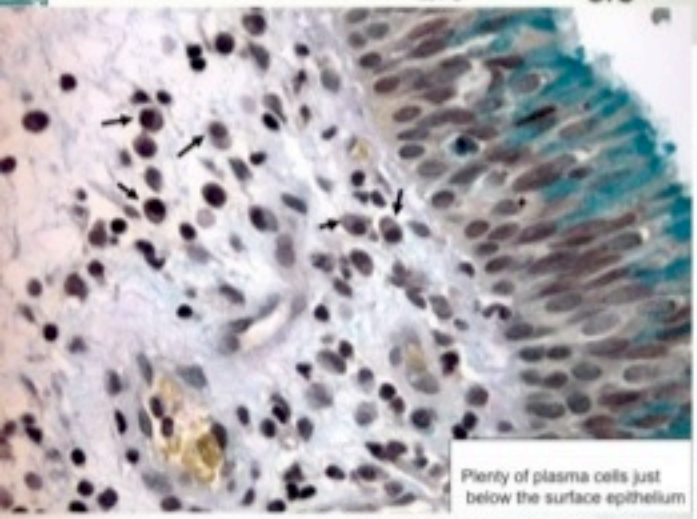
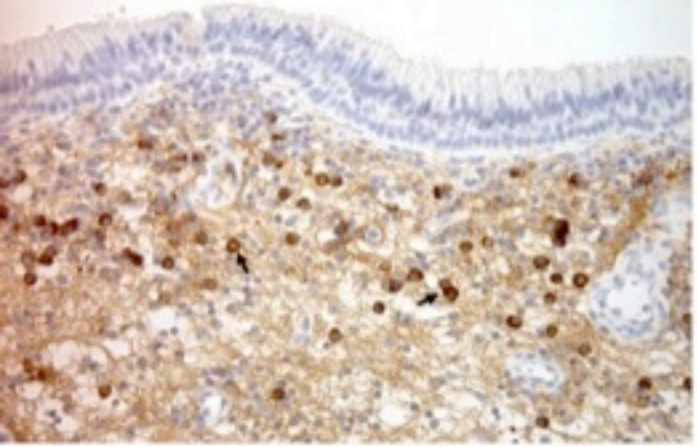
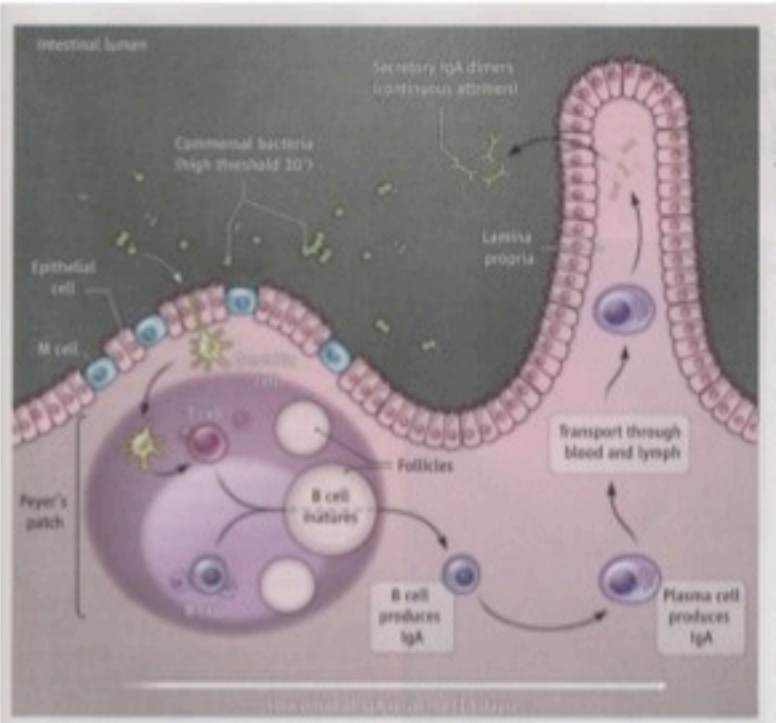




Immunoreaction to IgA in plasma cells in the mucosa (primary antibody diluted 1/100)

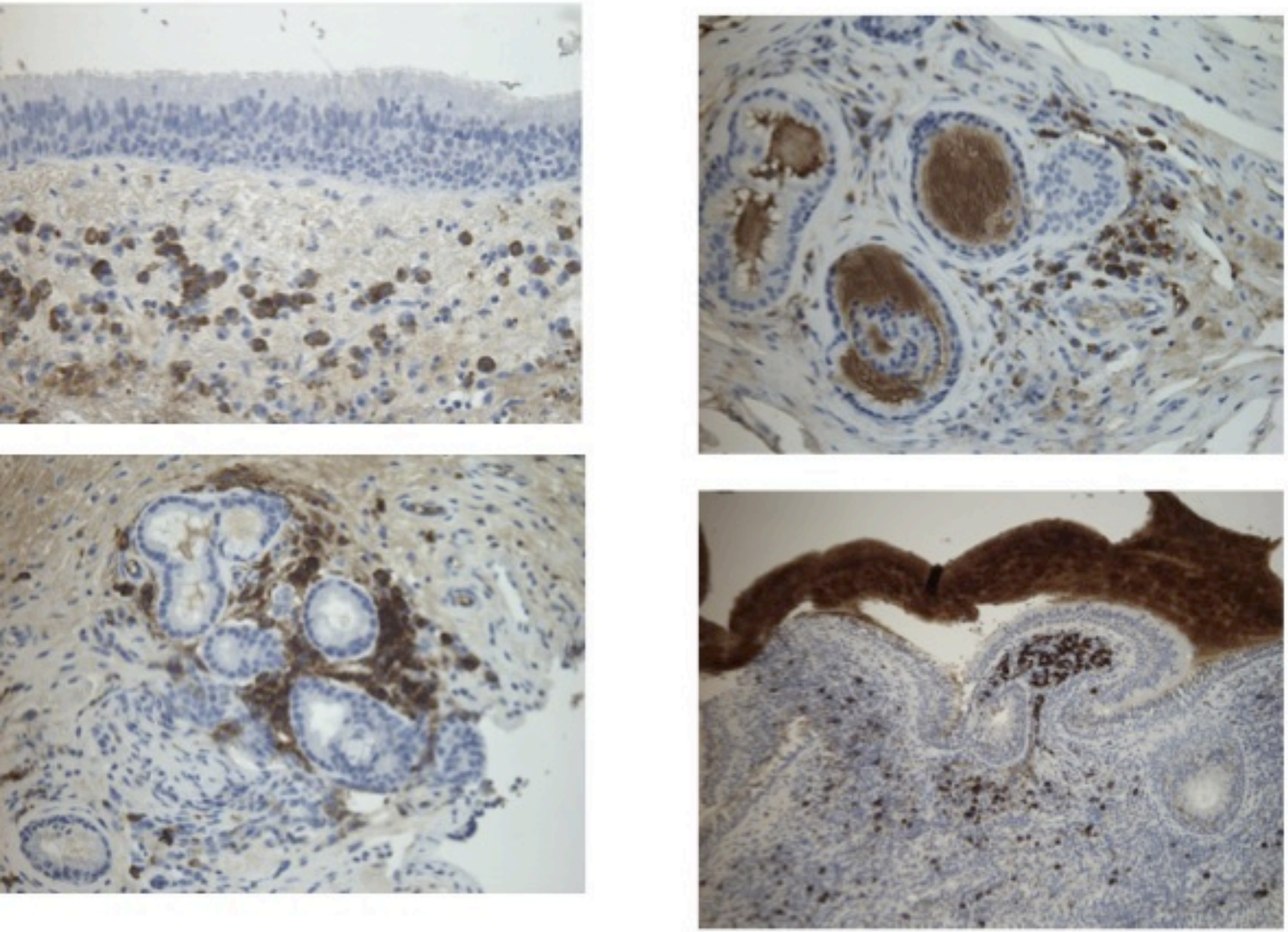




Intestinal IgA dynamics. Dendritic cells sample commensal bacteria from the intestinal lumen and migrate to follicles, where they present antigen to T cells. T cells and dendritic cells in the follicles stimulate B cells to mature and produce IgA. B cells then enter lymphatic and blood vessels. After about 14 days, they enter the lamina propria and differentiate into long-lived (more than 16 weeks) plasma cells that release IgA dimers. Dimers enter the intestinal lumen and function as secretory IgA. Exposure to a different commensal rapidly attenuates an ongoing response (attrition).

A. Cerutti: IgA changes the rules of memory. Science 328:1646-47; 2010

Mucosa Associated Lymphoid Tissus (MALT) – Sinus Associated Lymphoid Tissue (SALT)



Immunostaining: IgA producing plasmacells and IgA in submucosal glands and in mucus from paranasal sinus in a CF patient with sinusitis with mucoid and non-mucoid P. aeruginosa and intermittent P. aeruginosa colonization of the lungs

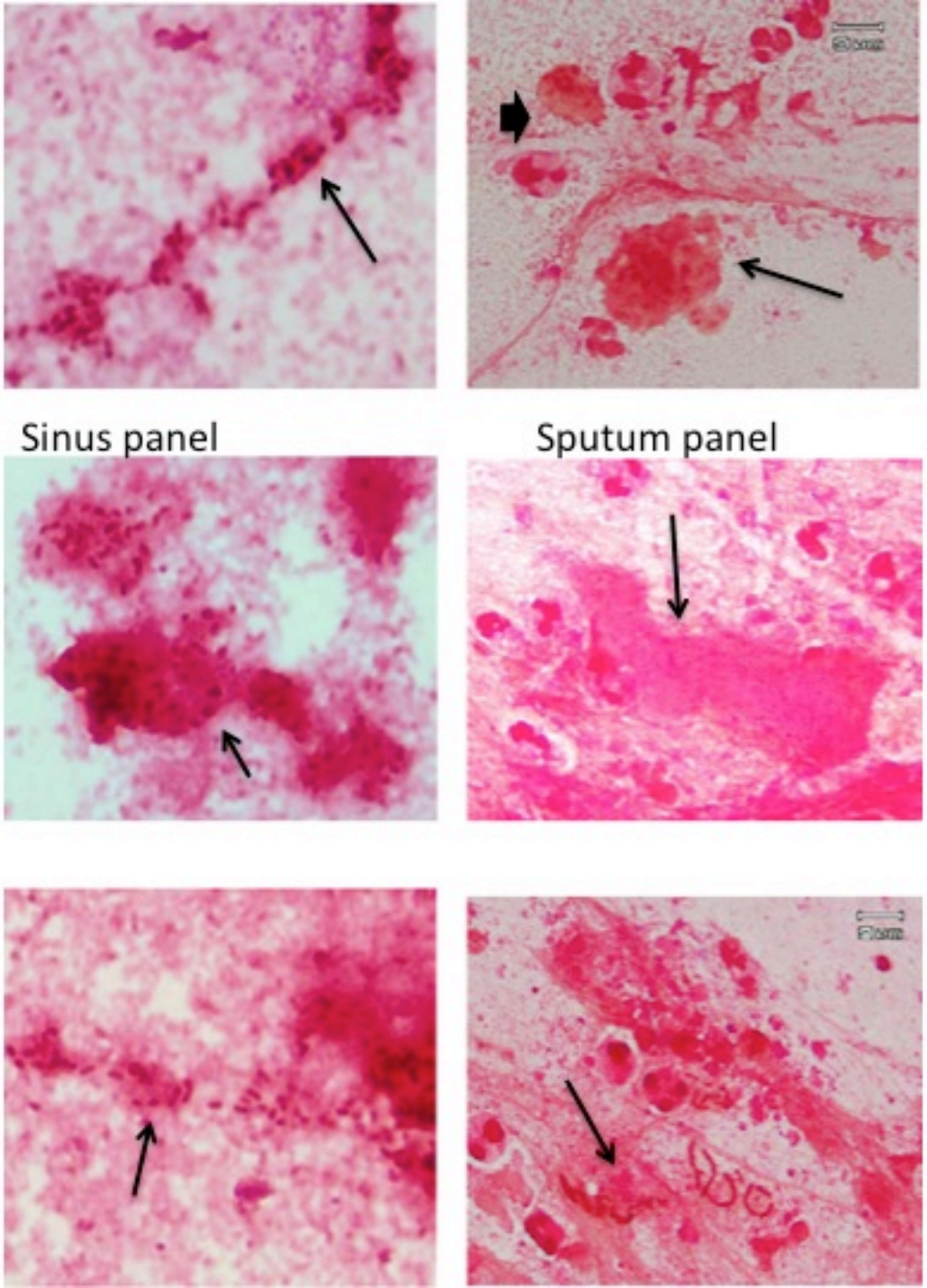
Pseudomonas-CF-IgG-ELISA® serum & modified for saliva & IgA (OD)

(Aanæs et all. Saliva IgA antibodies is a diagnostic tool for sinusitis caused by Pseudomonas aeruginosa in patients with cystic fibrosis. 34th European Cystic Fibrosis Conference, Hamburg, 8-11 June, 2011.)

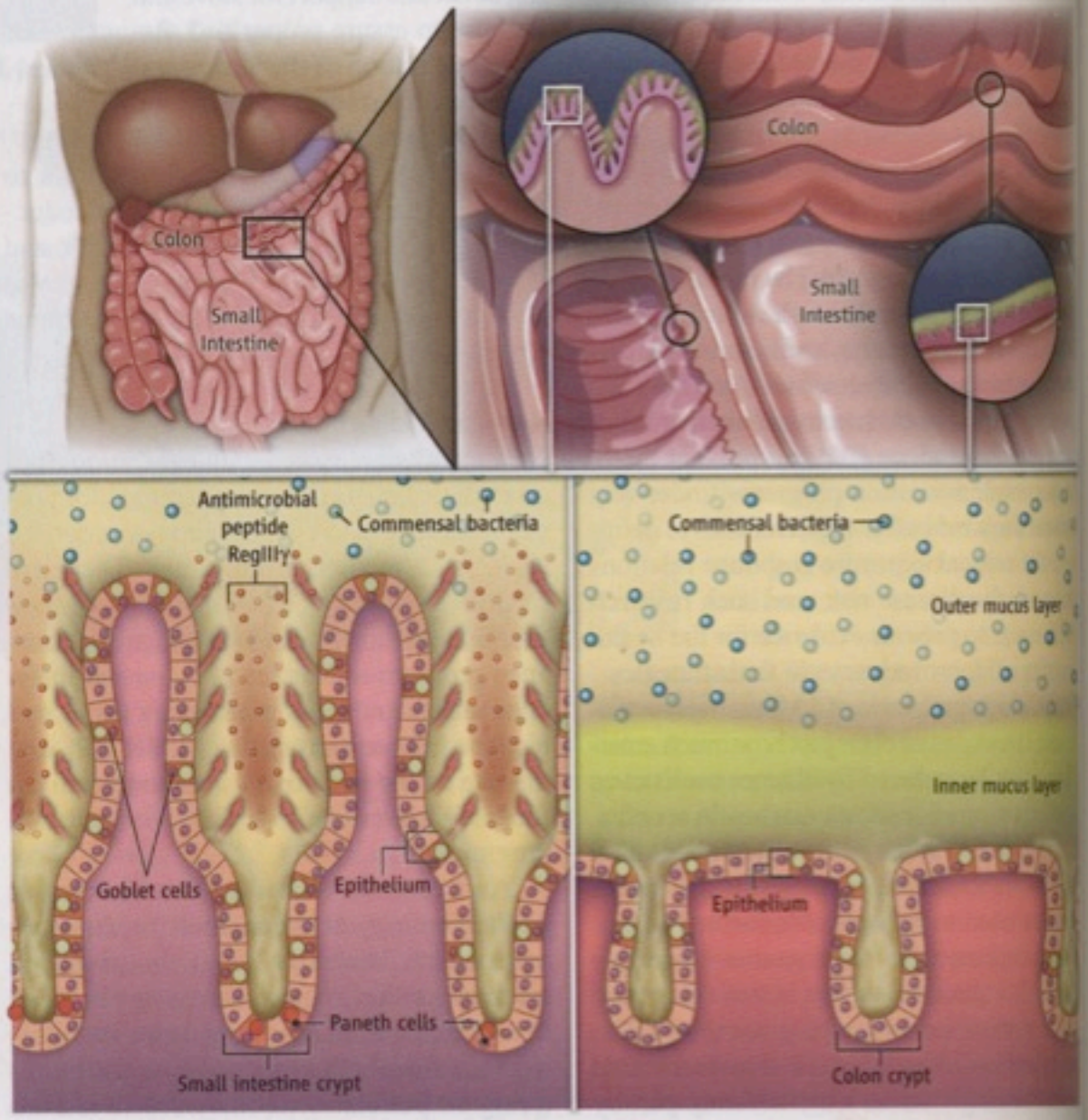
ALGINATE = BLUE ST-AG = RED	*Nasal IgA*	Serum IgA	Nasal IgG	Serum IgG	Nasal IgA/ Serum IgA	Nasal IgG/ Serum IgG	Nasal IgA / IgG	Serum IgA/ IgG
Non-CF Control group (9)	*0.17* *0.63*	0.05 0.51	0.09 0.28	0.12 0.96	3.29 1.74	0.92 0.24	2.19 2.93	0.57 0.56
CF free from Gram-neg. inf. (32)	*0.28** *0.77**	0.13 0.73*	0.09* 0.19*	0.12* 1.01*	2.57* 1.99	0.84* 0.19*	3.72 5.94*	1.24* 0.66
CF + intermit. PA (24)	*1.03** *1.33**	0.14* 1.21*	0.50* 0.64*	0.30* 1.68	27.92* 1.99*	2.80* 0.38	4.54 5.02*	0.68* 0.67
CF + chronic PA (25)	*1.70** *1.46*	0.45* 1.65	0.63* 0.96*	0.85* 2.52*	4.84* 1.06*	1.16 0.38	4.35 1.81*	0.82 0.66

* $p < 0.05$ when CF with intermittent P. aeruginosa lung colonization is compared with CF without Gram-negative lung infection or CF with chronic P. aeruginosa lung infection

Gram-stained smears from pus from sinus paranasales (left panel) and from corresponding sputum (right panel) from 3 CF patients with chronic P. aeruginosa infection treated with FESS. Biofilms are seen in all 6 photos (thin arrows). PMNs are plentifull in sputum (thick arrow) but scarce or absent in sinus pus.



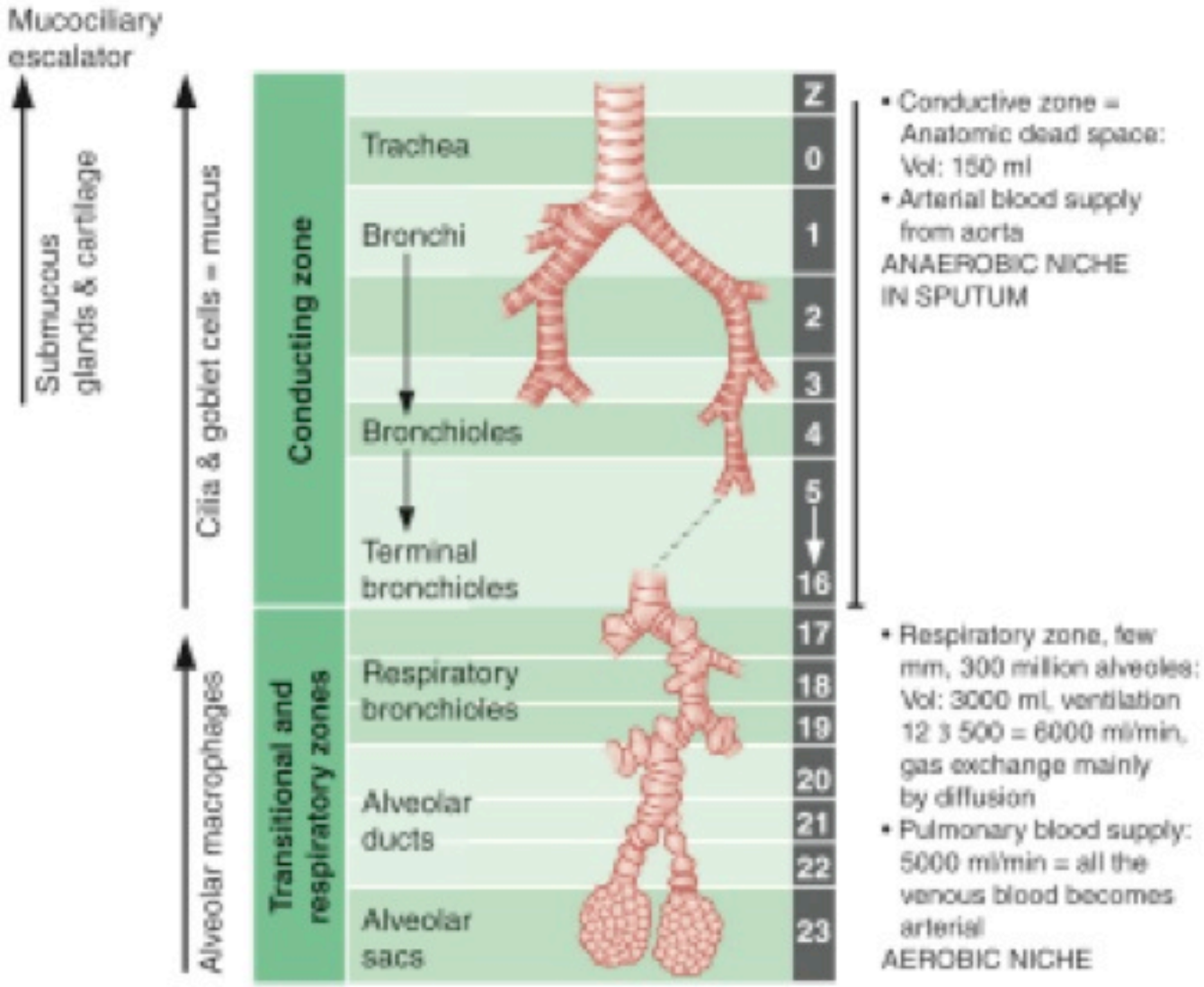
(Johansen, Aanæs, Pressler, Nielsen, Fisker, Skov, Høiby, Buchwald: Colonization and infection of the paranasal sinuses in cystic fibrosis patients is acompanied by a reduced PMN response. J. Cystic Fibrosis 2012, in press. Doi:10.1016/j.jcf.2012.04.011)



Intestinal protection. The intestinal epithelium is covered by mucus. In the colon, the inner stratified mucus layer acts as a physical barrier separating bacteria from the epithelium. The commensal bacteria are kept at a distance in the penetrable outer mucus layer. The small intestine is only covered with penetrable mucus where antibacterial molecules such as RegIIIγ generate a bacteria-depleted region close to the epithelium.

Johansson & Hansson: Keeping bacteria at a distance. Science 334:182-183; 2011

FIGURE 3

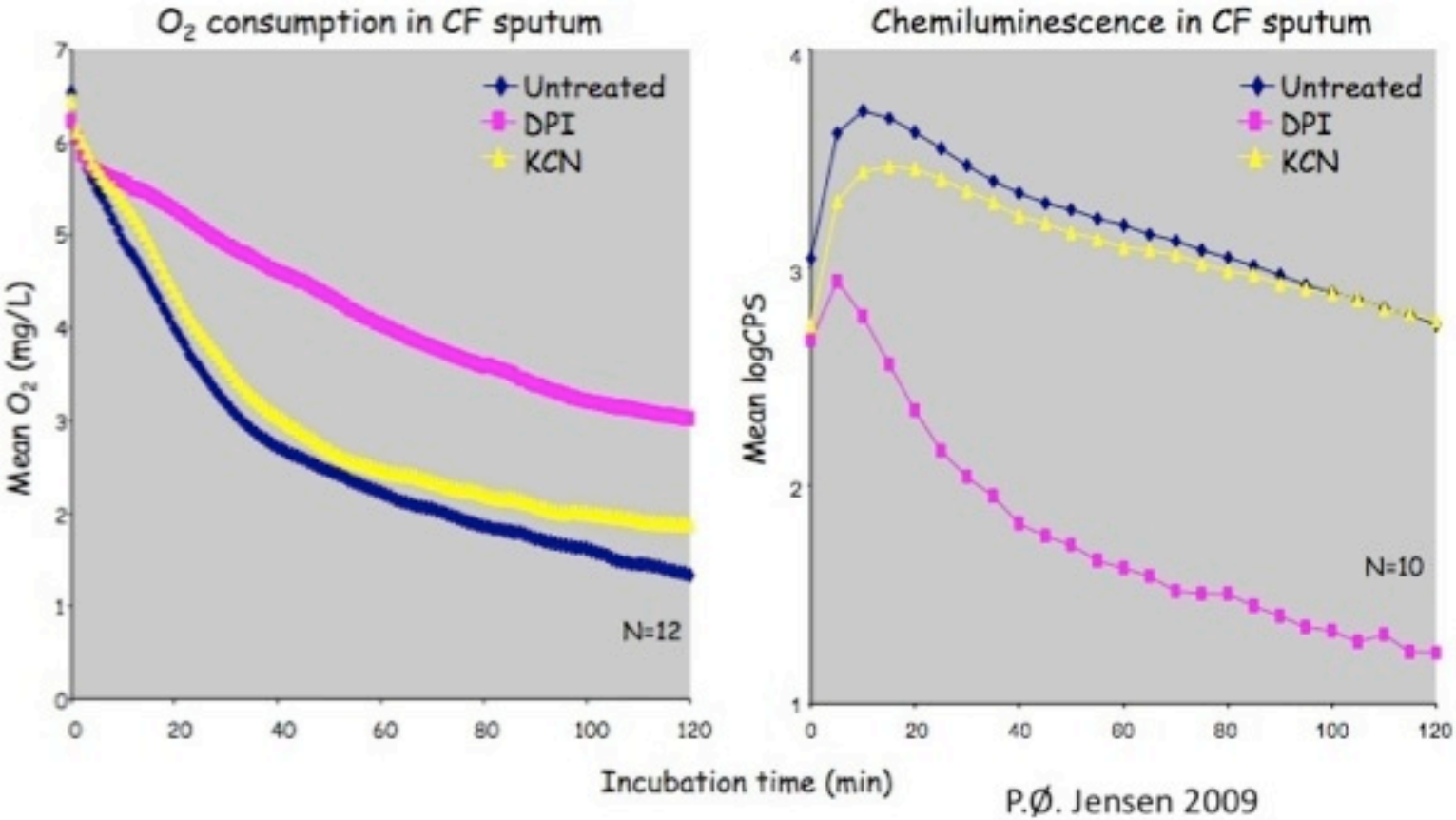


The conductive and respiratory zone of the lungs.

Respiratory Burst of PMNs in Sputum from CF Patients

Fate of oxygen during the interaction between PMNs and *P. aeruginosa* in CF

CF sputum is bio-active with O_2 consumption by the respiratory burst and aerobic respiration



DPI inhibits PMN NADPH oxidase

KCN inhibits *P. aeruginosa* cytochrome

(Kolpen et al: Polymorphonuclear leukocytes consume oxygen in sputum from chronic Pseudomonas aeruginosa pneumonia in cystic fibrosis. Thorax 65:57-62; 2010.)

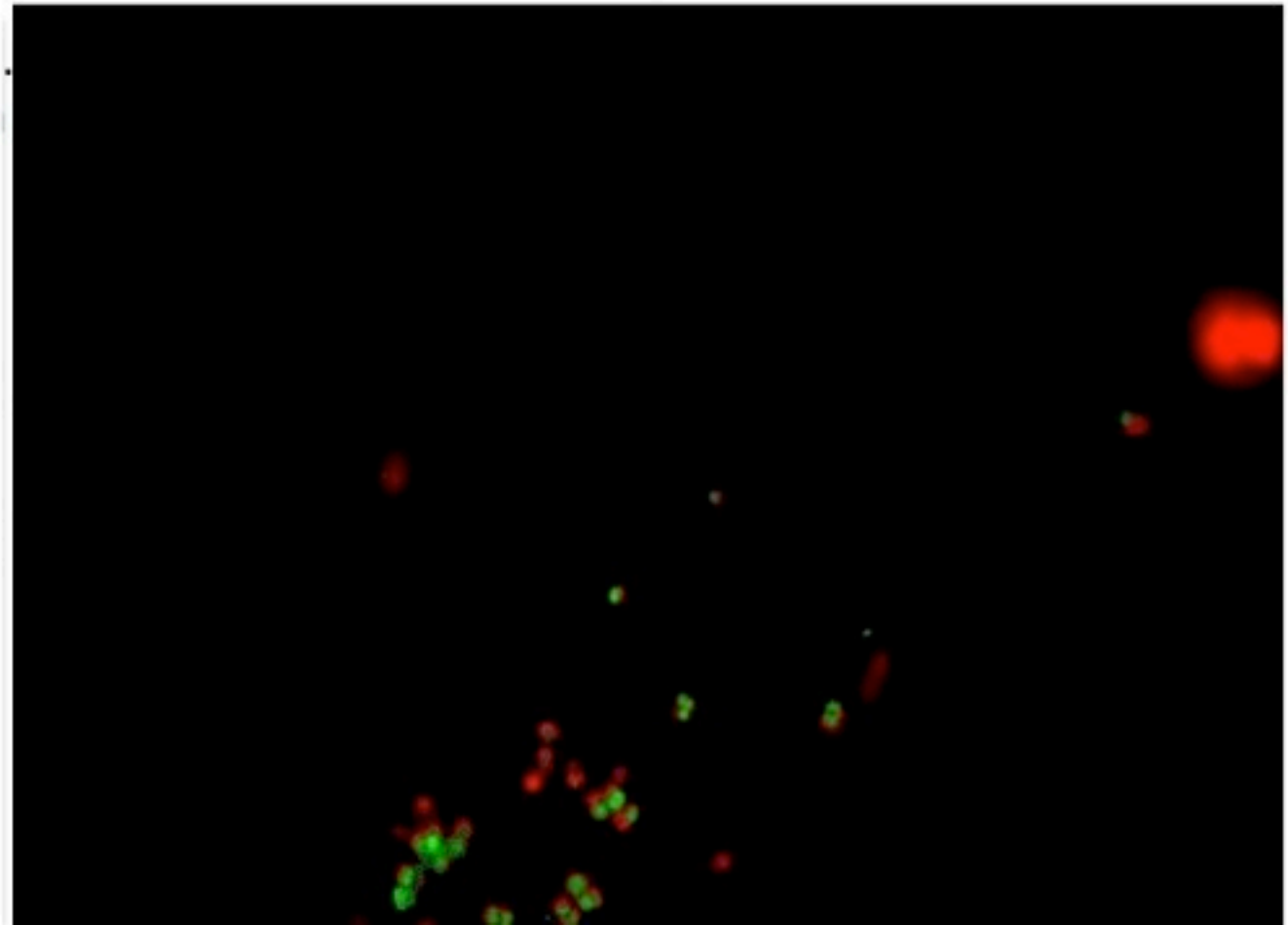
Respiratory Burst of PMNs in Sputum from CF Patients

Fate of oxygen during the interaction between PMNs and *P. aeruginosa* in CF

Ongoing ·NO-production by PMNs in CF sputum

Visualization of ·NO - production with DAF-FM (NO-indicator) and DNA with Syto59 in CF sputum

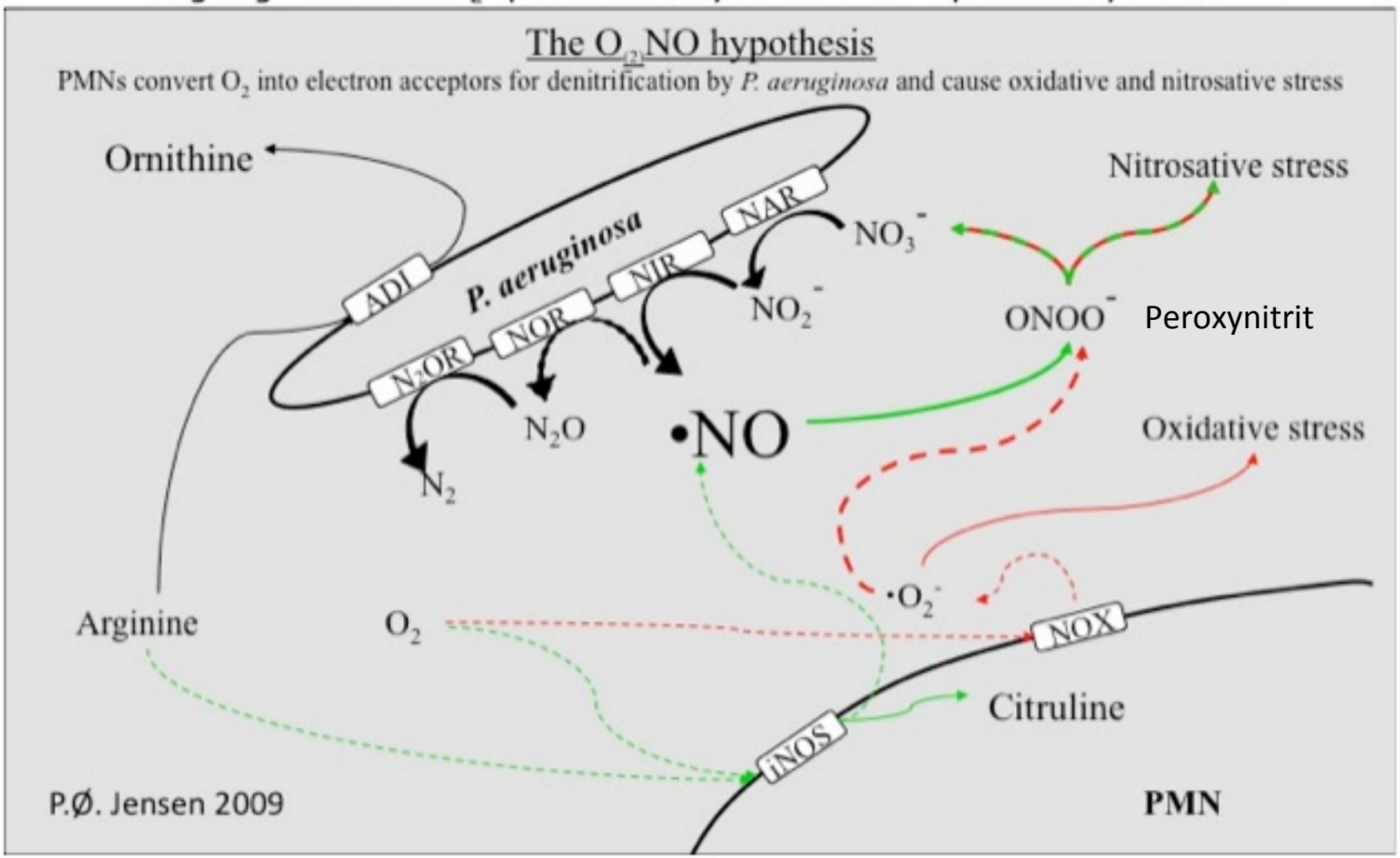
(Kolpen et al: Polymorphonuclear leukocytes consume oxygen in sputum from chronic Pseudomonas aeruginosa pneumonia in cystic fibrosis. Thorax 65:57-62; 2010.)



Respiratory Burst of PMNs in Sputum from CF Patients

Fate of oxygen during the interaction between PMNs and *P. aeruginosa* in CF

Ongoing ·NO and ·O_2^--production by PMNs in CF sputum may result in:



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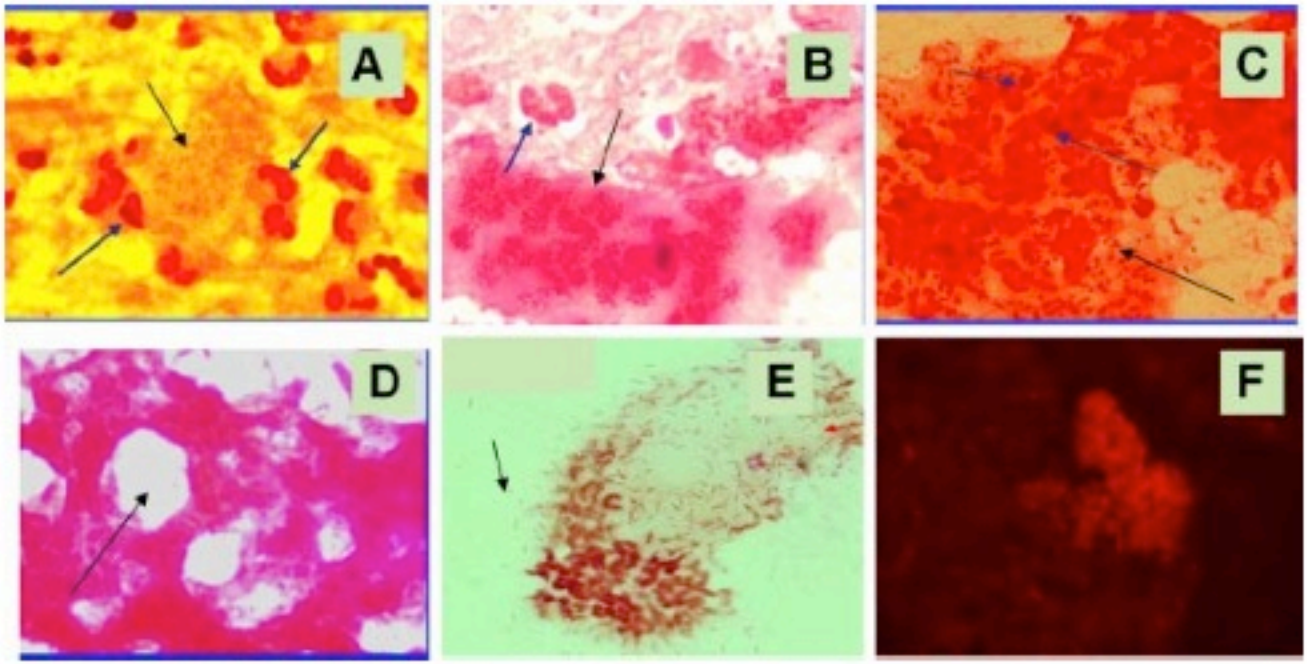
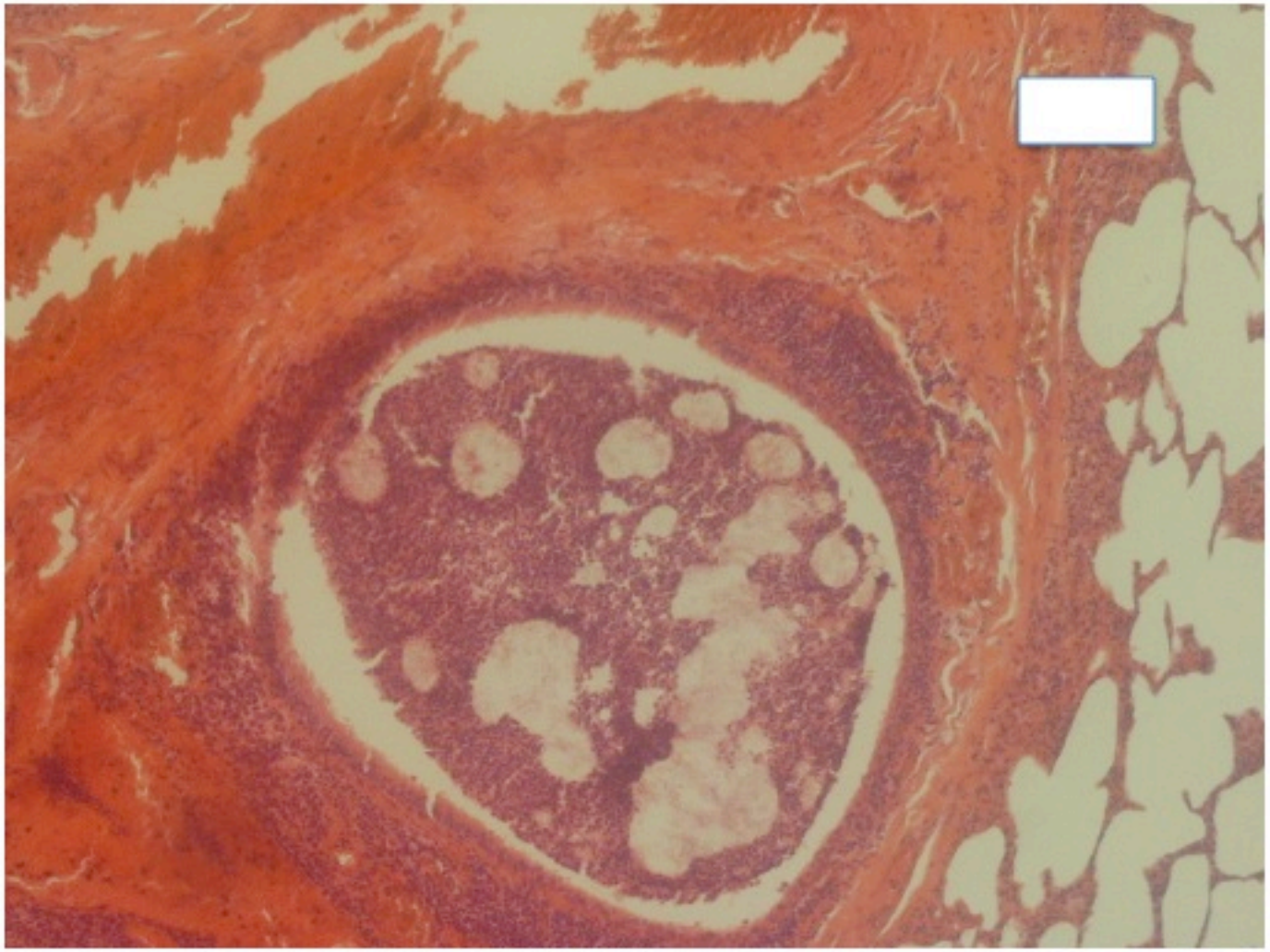
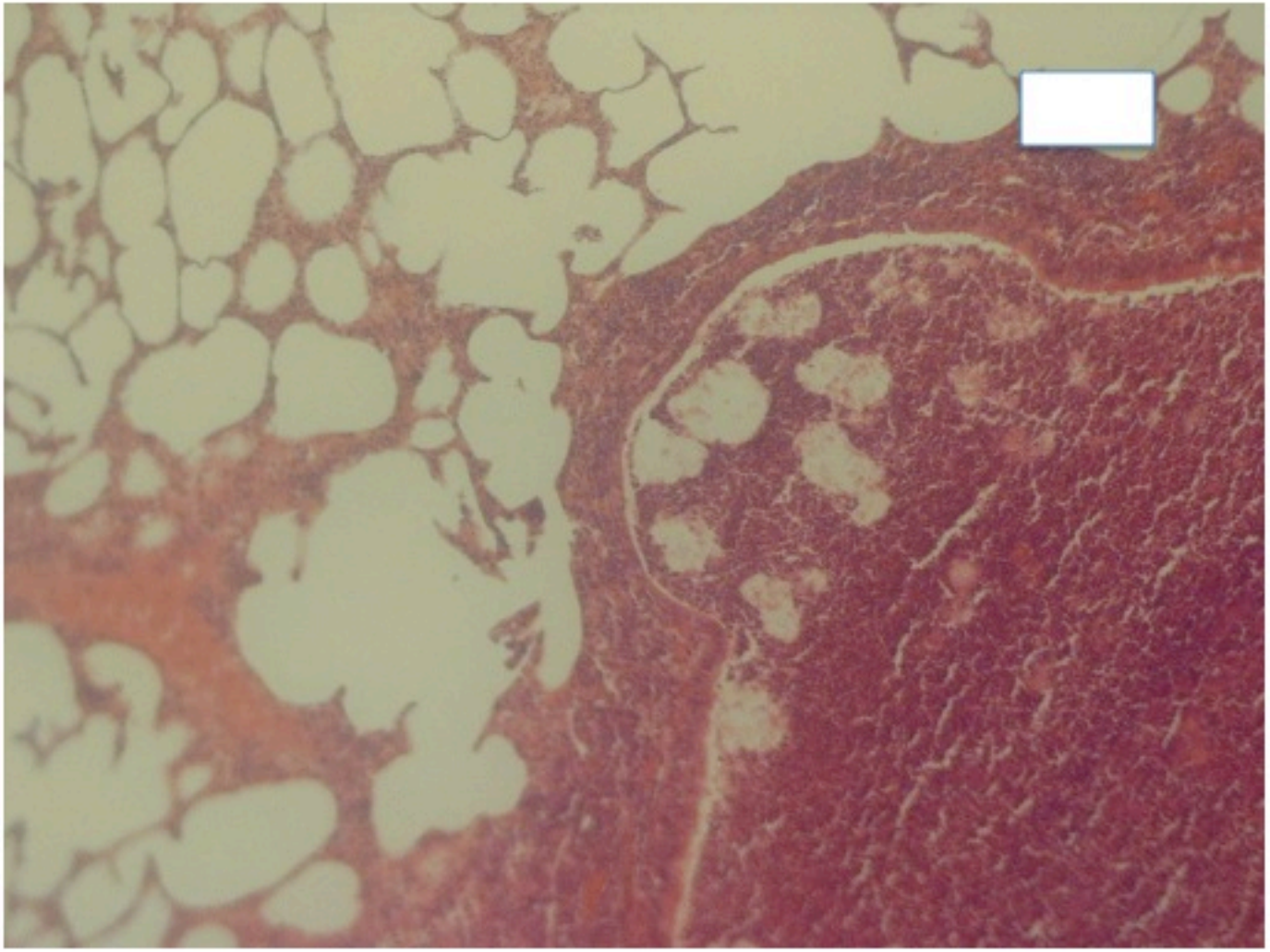


Fig. 1. Diversity of *P. aeruginosa* biofilms in sputum. Sputum samples from 77 chronic *P. aeruginosa* infected CF patients were examined by Gram stain and PNA FISH. The exclusive presence of *P. aeruginosa* were verified by standard culturing and *P. aeruginosa* specific PNA FISH (F). A great diversity of the organization of *P. aeruginosa* was detected, both compact biofilm forming microcolonies (frame A, B, C, F (blue arrows point at leukocytes, black arrows point at bacterial aggregates)) and non-adhered planktonic bacteria (E (arrow)) were observed. The organization of the PMNs was also diverse with PMNs surrounding the biofilms (A), distant from the biofilms (B) and in a very few cases appeared inside the biofilms (C). The biofilm microcolonies were mostly very compact, however some samples were perforated with "holes" mimicking water-filled channels visible in some mature in vitro biofilms[51] (D (arrow point at hole)).

(Bjarnsholt, T., Jensen, P.Ø., Fiandaca, M.J., Pedersen, J., Hansen, C.R., Andersen, C.B., Pressler, T., Givskov, M., Høiby, N.: Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. Pediatric Pulmonology. 44:547-558; 2009)



Høiby 2012



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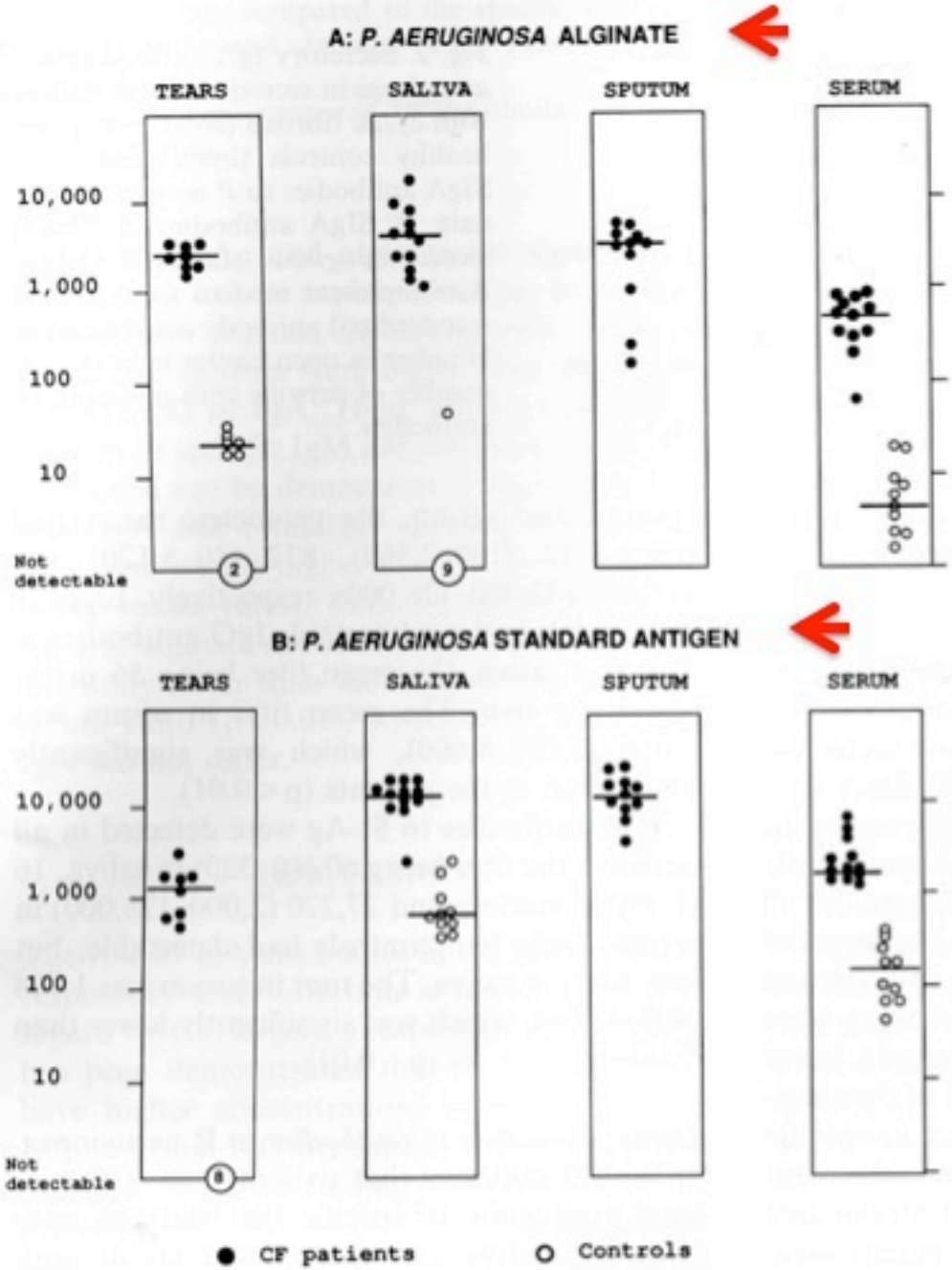

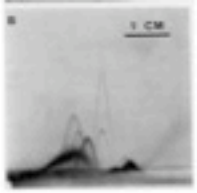

13 CF PATIENT WITH CHRONIC P. AERUGINOSA INFECTION, 10 HEALTHY CONTROLS.

(Human colostrum as standard, HRP-rabbit IgG against human α-chain (IgA))

Fig. 1. IgA antibodies to *P. aeruginosa* in secretions and serum from patients with cystic fibrosis (solid circles) and healthy controls (open circles). A: Antibodies to *P. aeruginosa* alginate. B: Antibodies to *Pseudomonas aeruginosa* standard antigen. Bars represent median values of the standardized antibody concentration. Number in open circles indicates the number of persons with undetectable antibodies.

Pedersen, Møller, Espersen, Sørensen, Jensen, Høiby: Mucosal immunity to P. aeruginosa alginate in cystic fibrosis. APMIS 100:326-34; 1992.

Høiby 2009

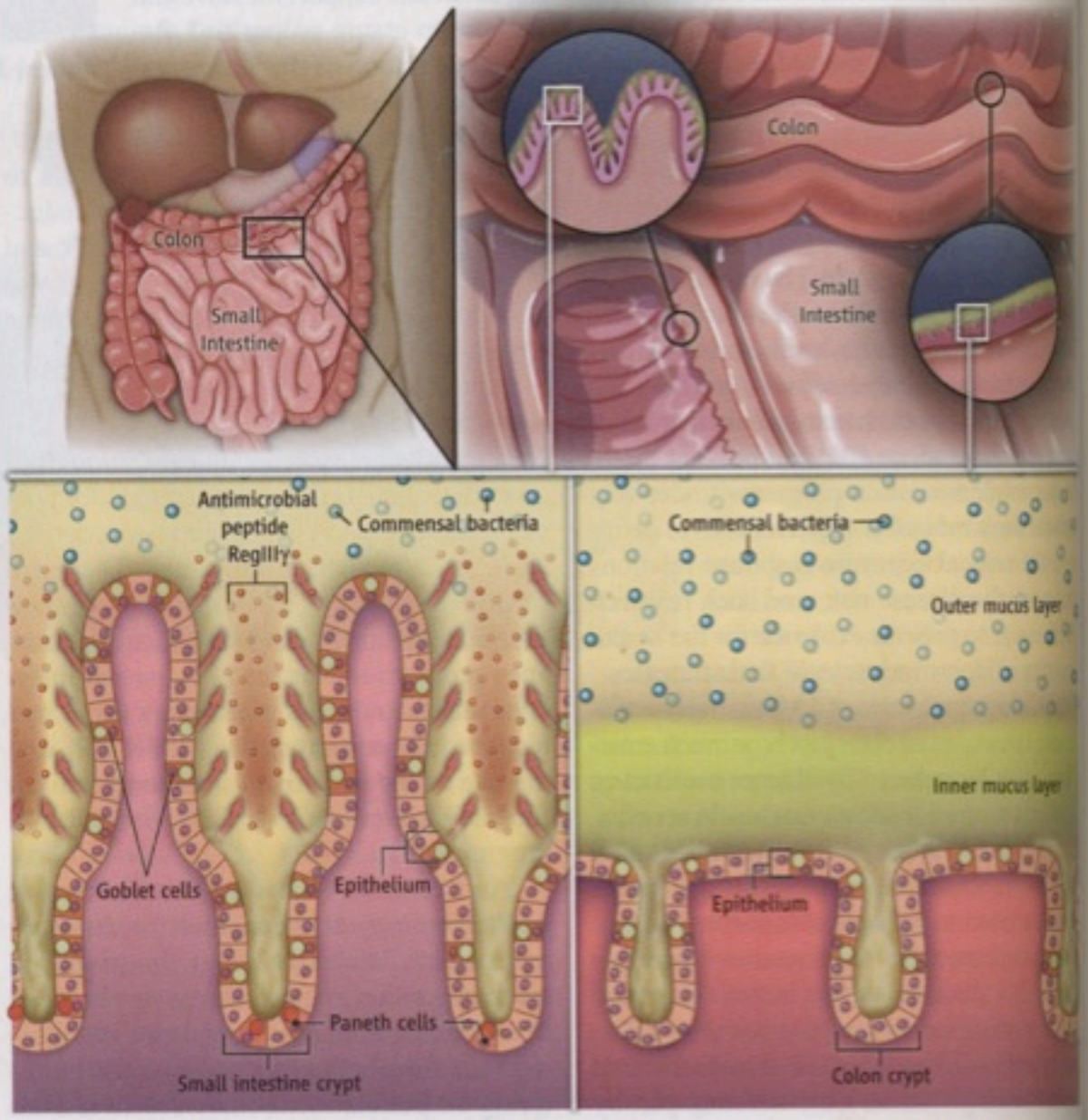

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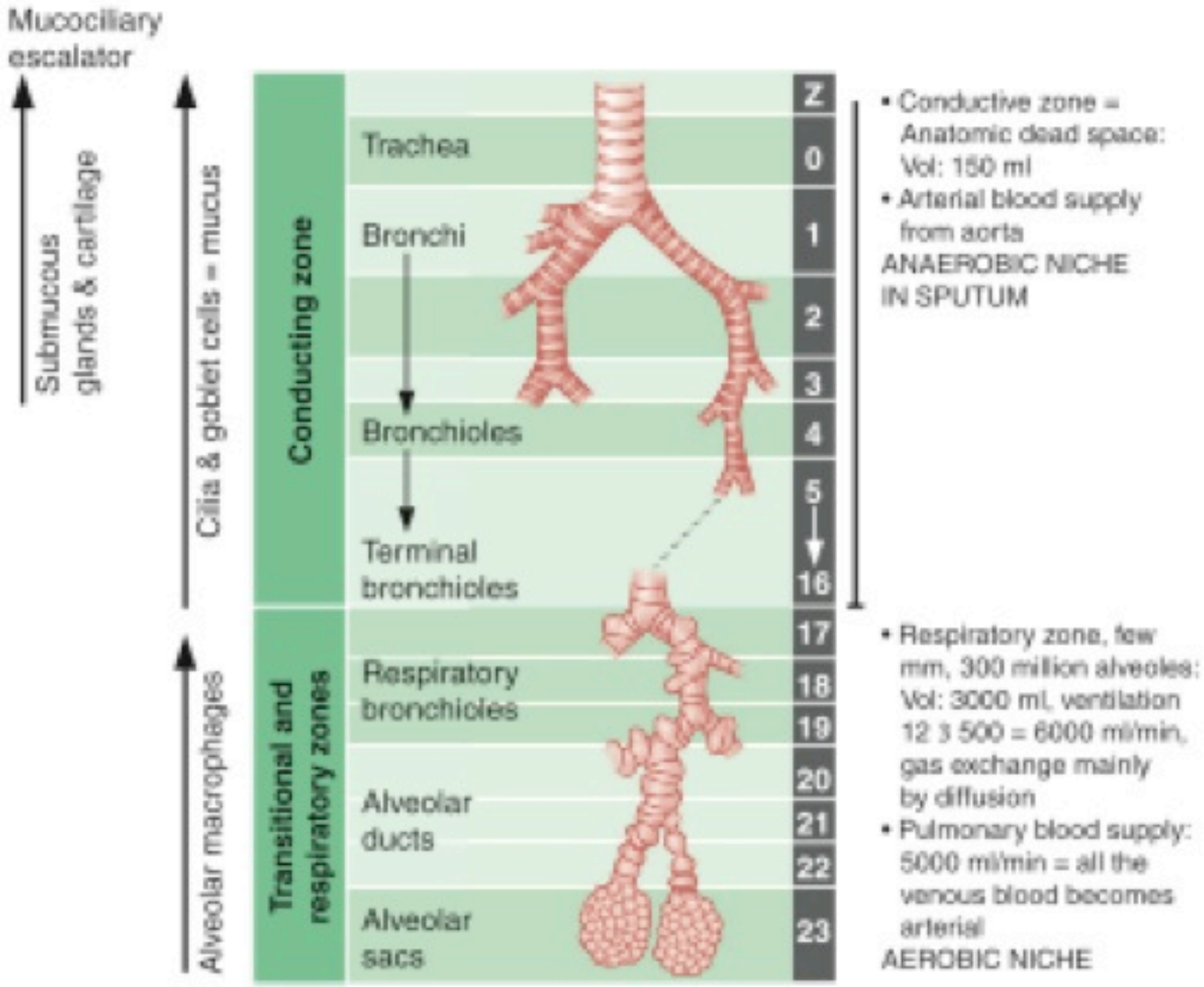
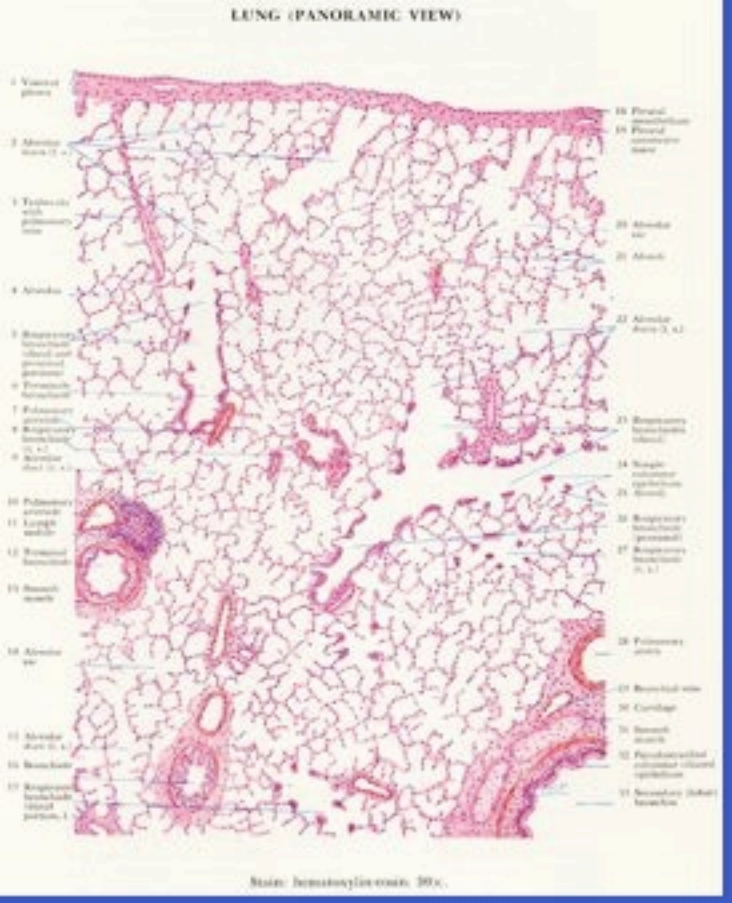
FIGURE 3



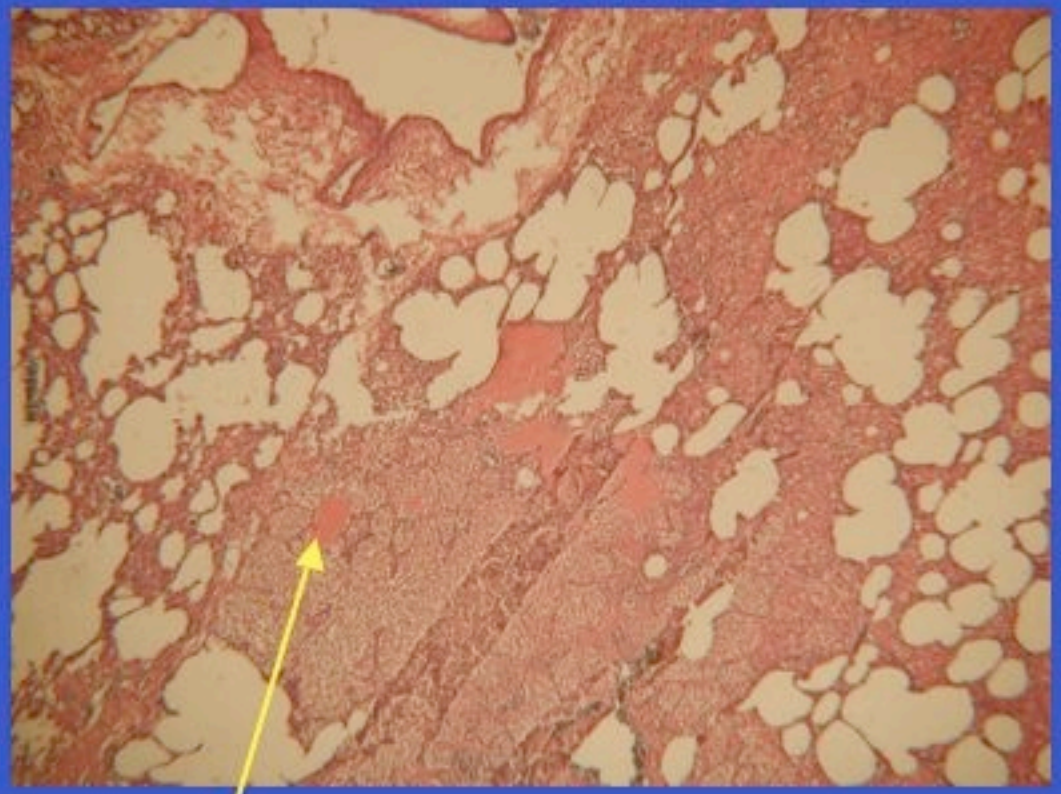
The conductive and respiratory zone of the lungs.

Høiby 2012



Normal lung

The *packed* aerobic CF alveolar incubation chamber *filled* with mucoid P. aeruginosa biofilm

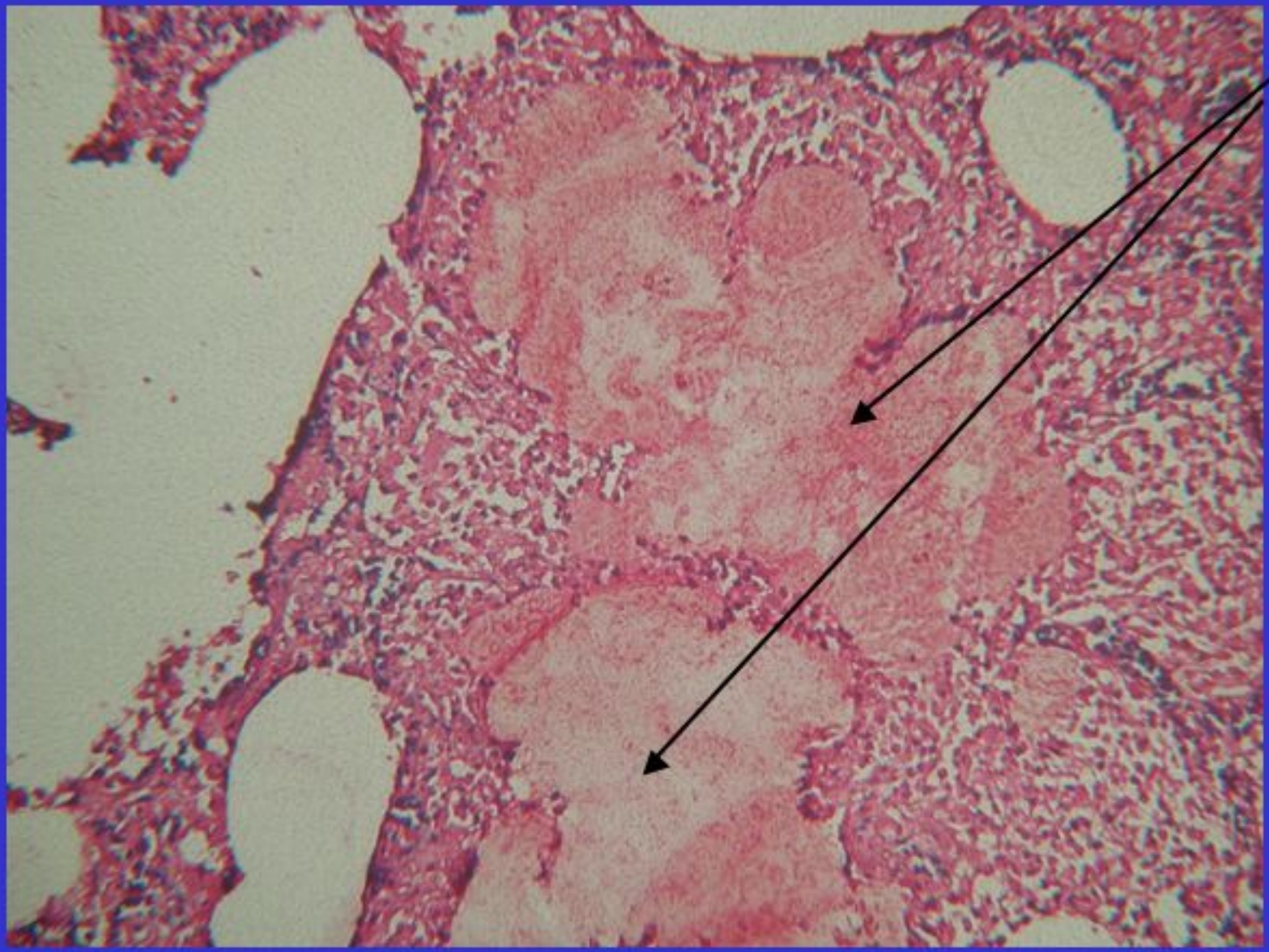


Autopsy (BS242/74) of a Danish CF girl (MLM) who died due to chronic P. aeruginosa lung infection and 21 precipitating antibodies against P. aeruginosa (normal: 0-1). Severely inflammed tisssue (pneumonia). HE stain x 40.

HØIBY 2004

IgG response!

The *packed* aerobic CF alveolar incubation chamber *filled* with mucoid P. aeruginosa biofilm



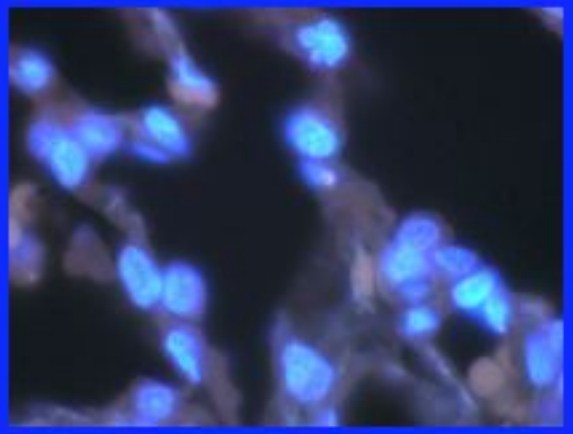
Mucoid biofilm of P. aeruginosa in an alveolar sac surrounded by severely inflammed tissue (pneumonia)

HØIBY 2004

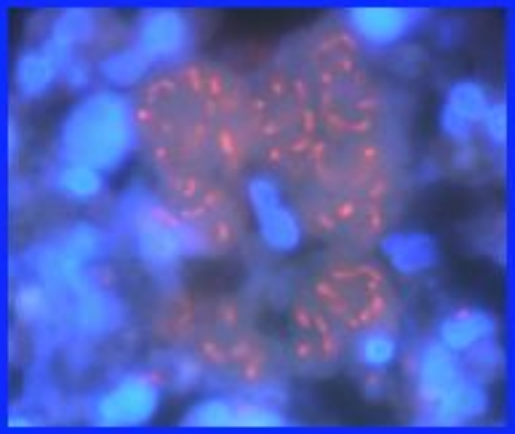
Autopsy (BS242/74) of a Danish CF girl (MLM) who died due to chronic P. aeruginosa lung infection and 21 precipitating antibodies against P. aeruginosa. HE stain x 100.

Explanted lung from CF patient with chronic P. aeruginosa infection, biofilms, PNA-FISH and DAPI stains

Alveoles

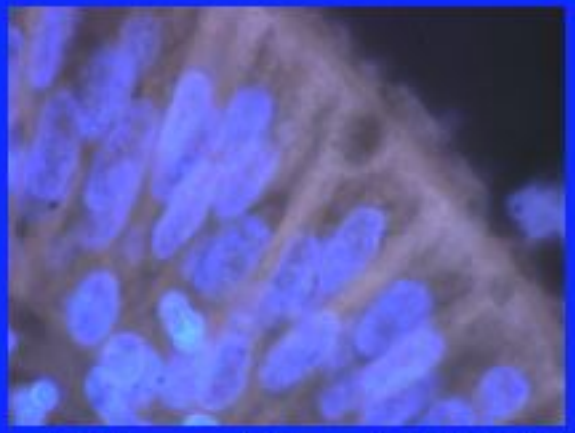


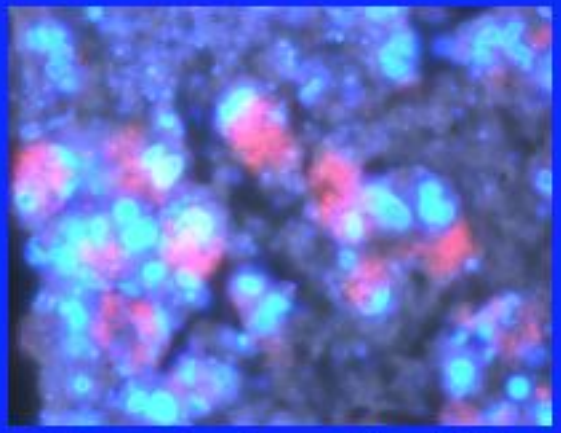
CF 1312 02



PA biofilm

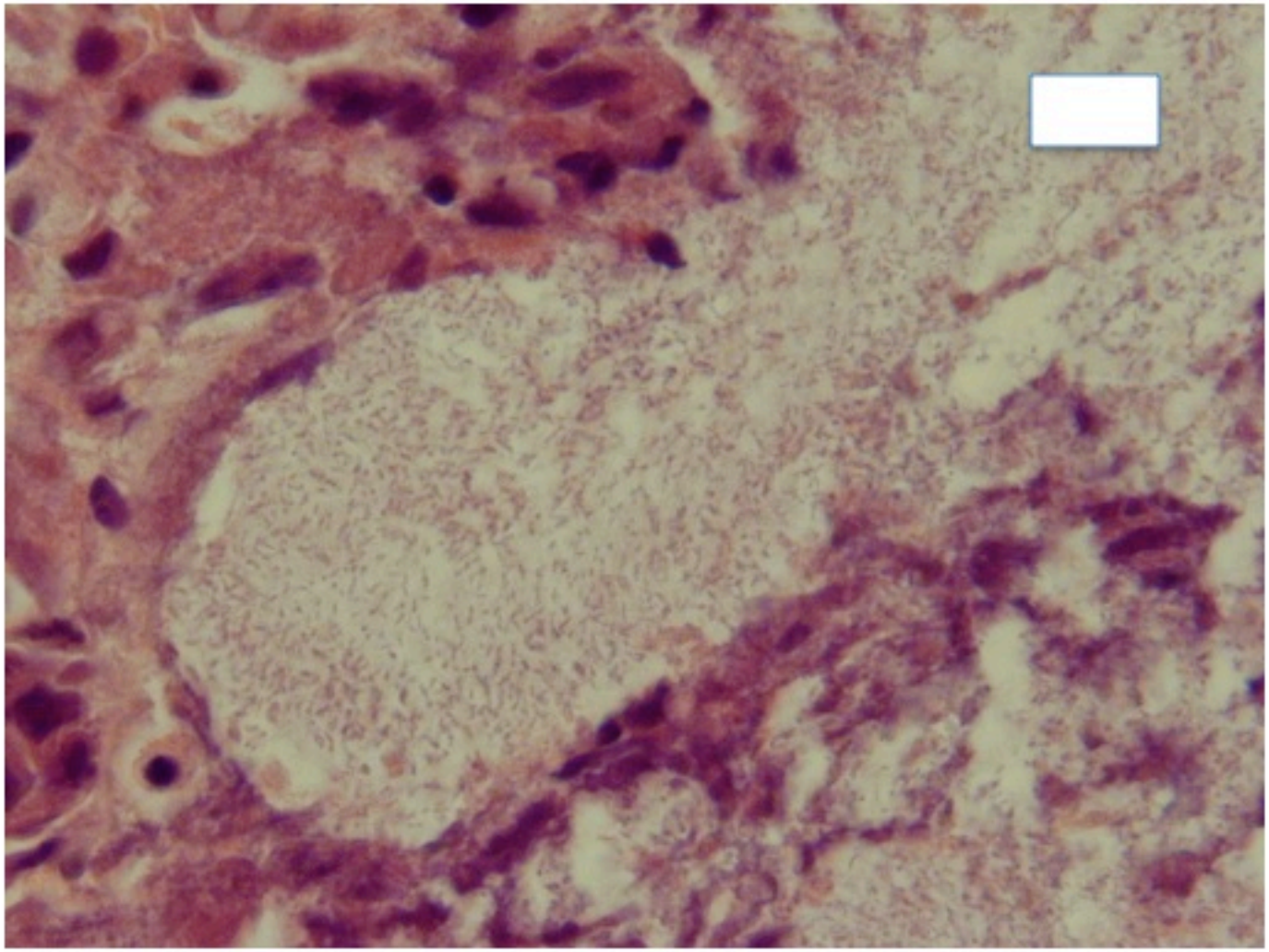
Ciliated epithelial cells



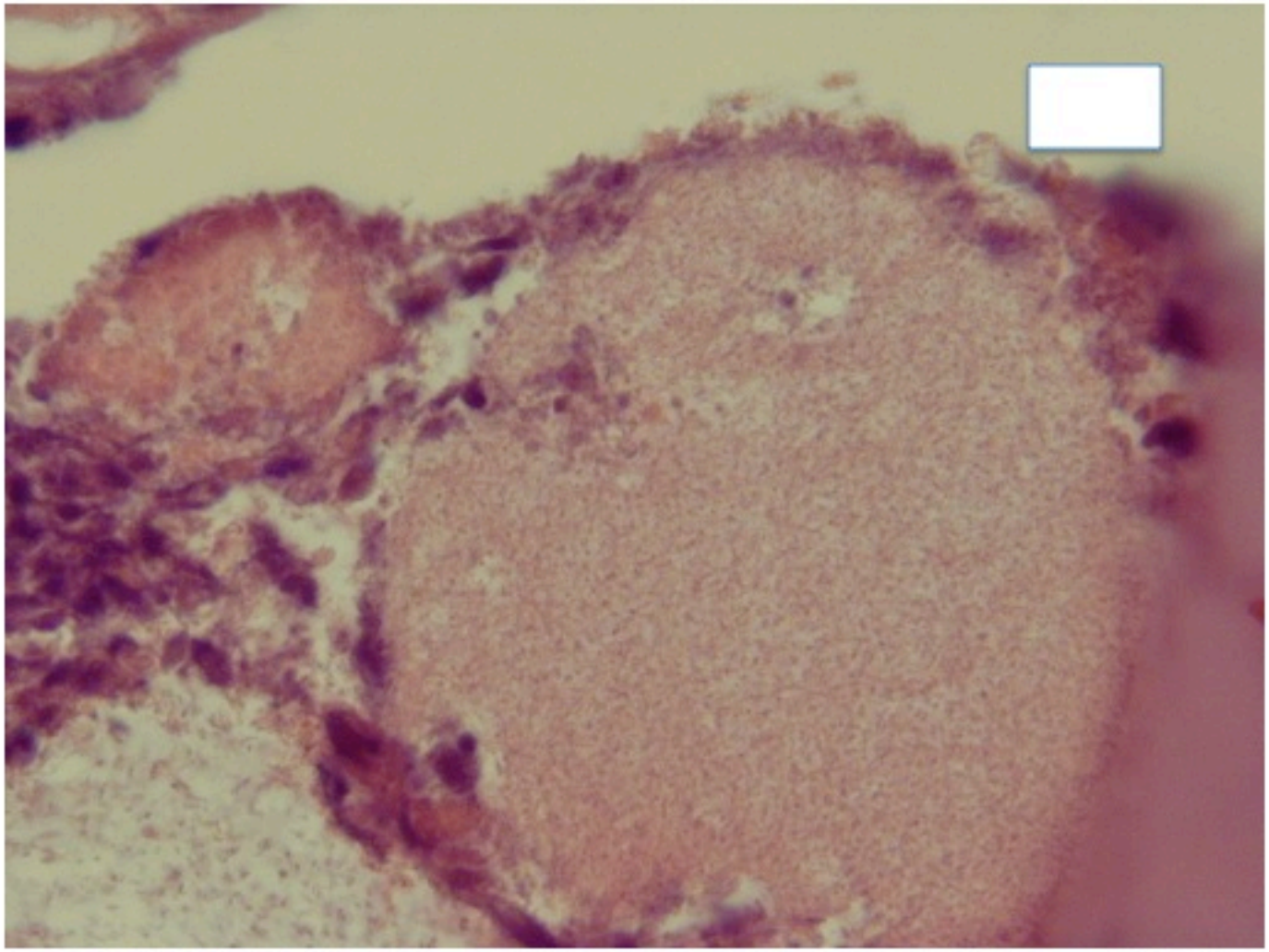


PMNs with phagocytosed NM PA

(Courtesy of Thomas Bjarnsholt 2007: 'The P. aeruginosa burden of the CF lung - uncovering the black box')



Høiby 2012



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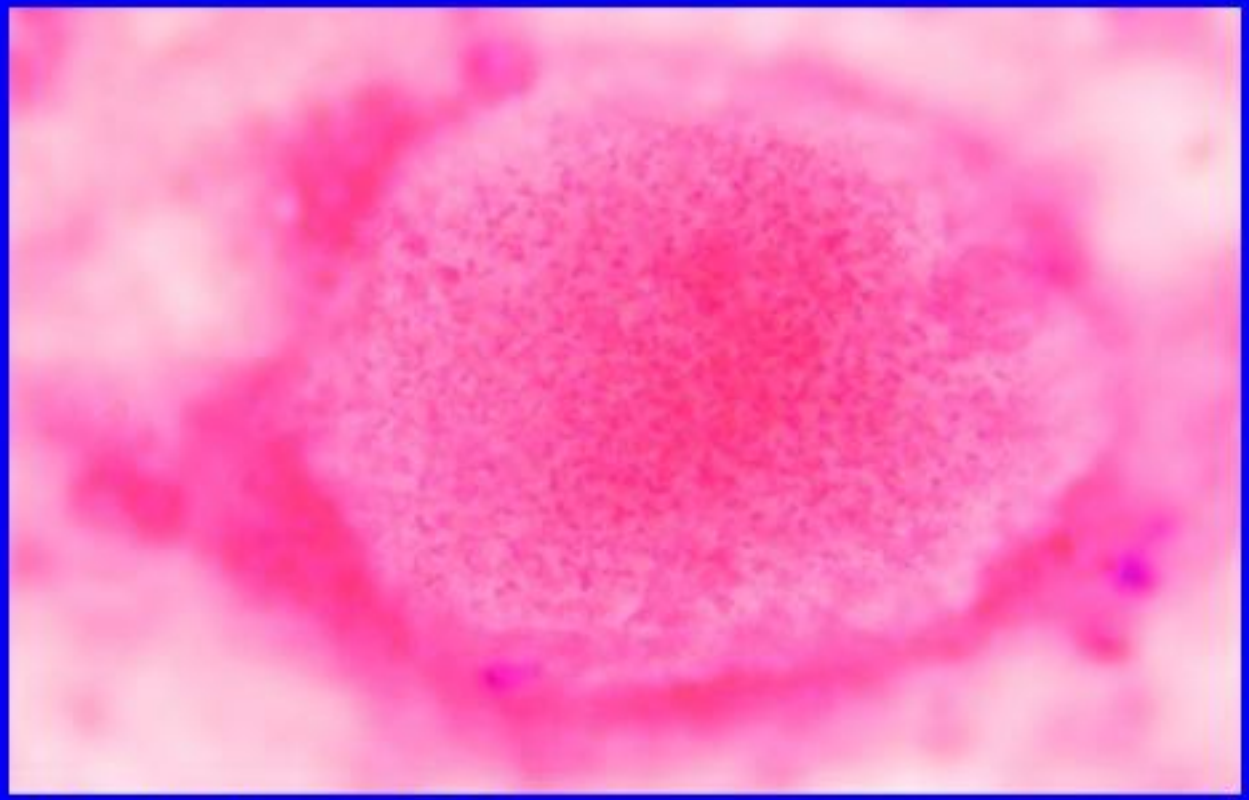
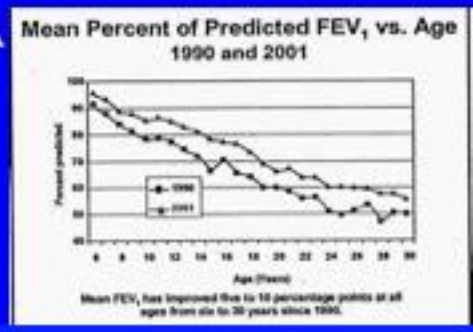


Gram-stained sputum, CF 71, female 44 y, mucoid & non-mucoid PA.

PA since 1970, chronic mucoid PA since 1971, 35-44 precipitaitng antibodies against PA for many years. A detached alveolus with a PA biofilm is seen (x100 & x1000). The healthy lung consists of 300,000,000 alveoles, 1-2% decline of pulmonary function/year means loss of 3-6 mio alveoles/year = loss of 2700 - 5400 alveoles/day. HØIBY 2005

(1) Bruce et al. Am.Rev.Respir.Dis. 132:529;1985., 2)Amnitzbøll et al. Acta Pæd. Scand. 77:842;1988)



The result of the *packed* CF incubation chamber *filled* with mucoid P. aeruginosa biofilm = FOCAL inflammatory- mediated tissue destruction!

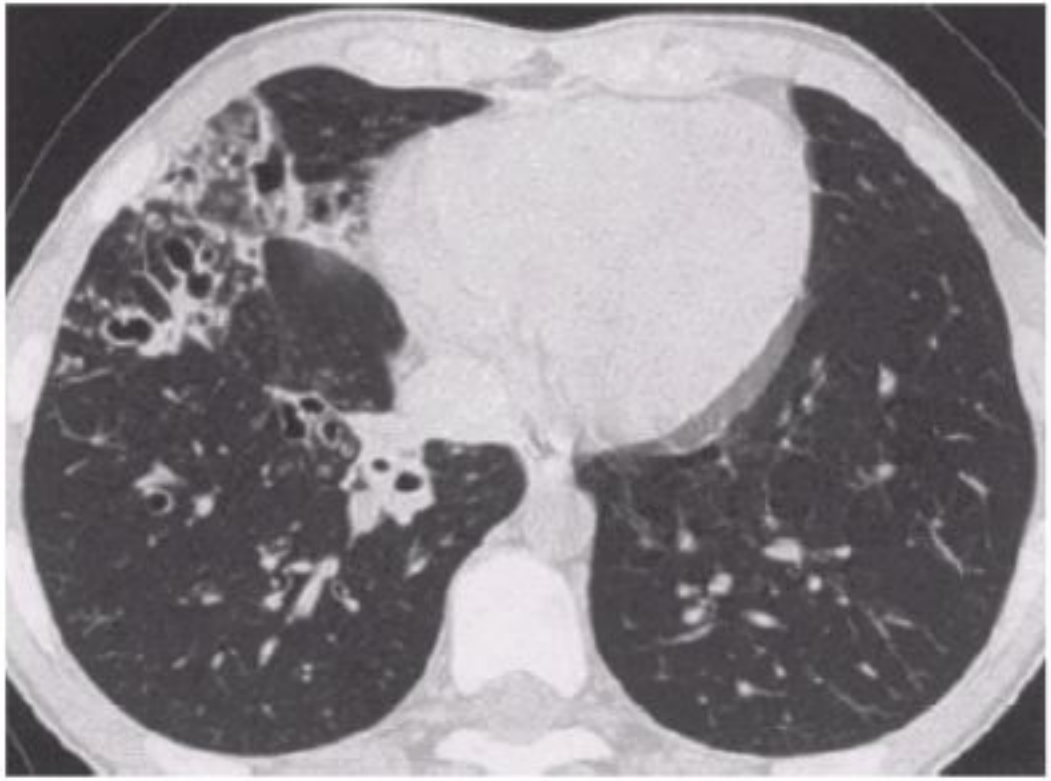
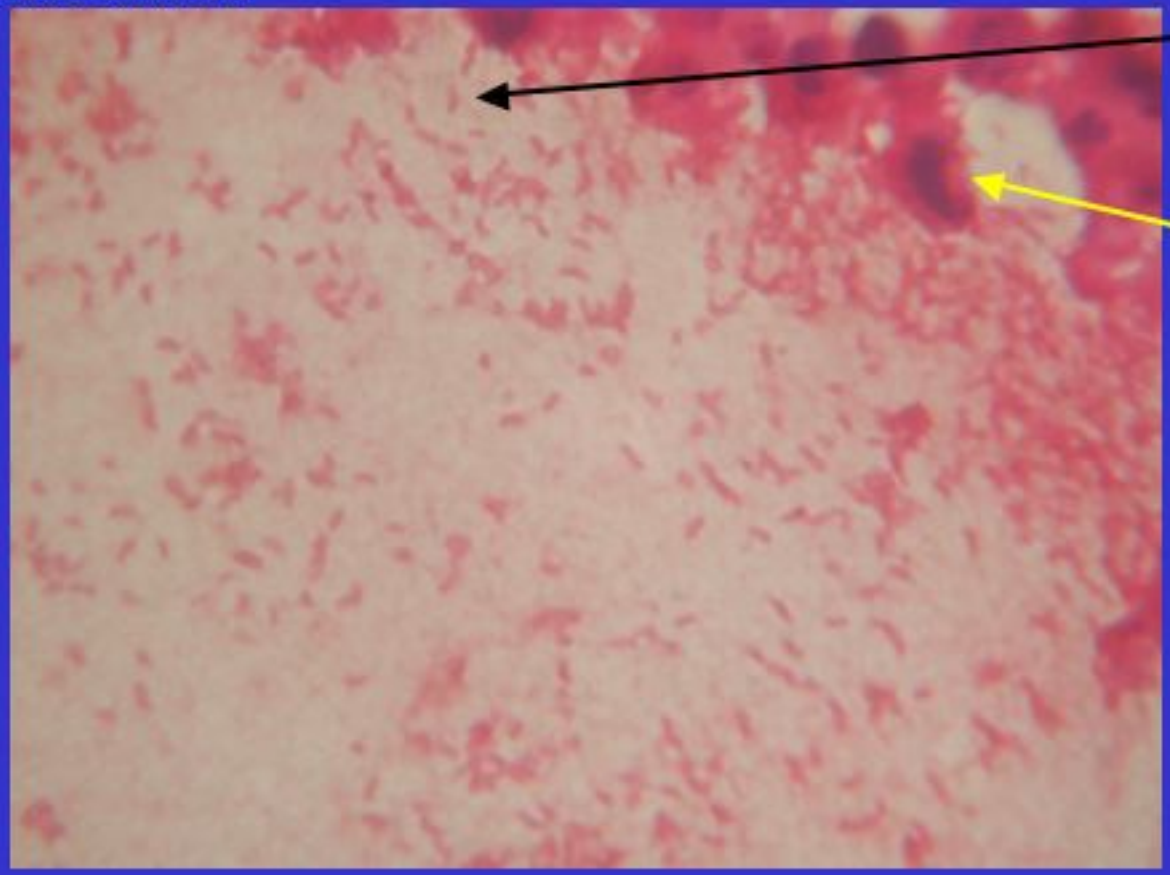


Fig. 2. HRCT scan of 13-year-old boy with normal function (FEV_1, 99%; FVC, 92%; FEV_1/FVC ratio, 90%; FEF_{25-75}, 95%) but with localized areas of "end"-stage lung disease.

HØIBY 2004

(Tiddens. Ped. Pulmonol. 34;228-31; 2002)

The *packed* aerobic CF alveolar incubation chamber *filled* with mucoid P. aeruginosa biofilm

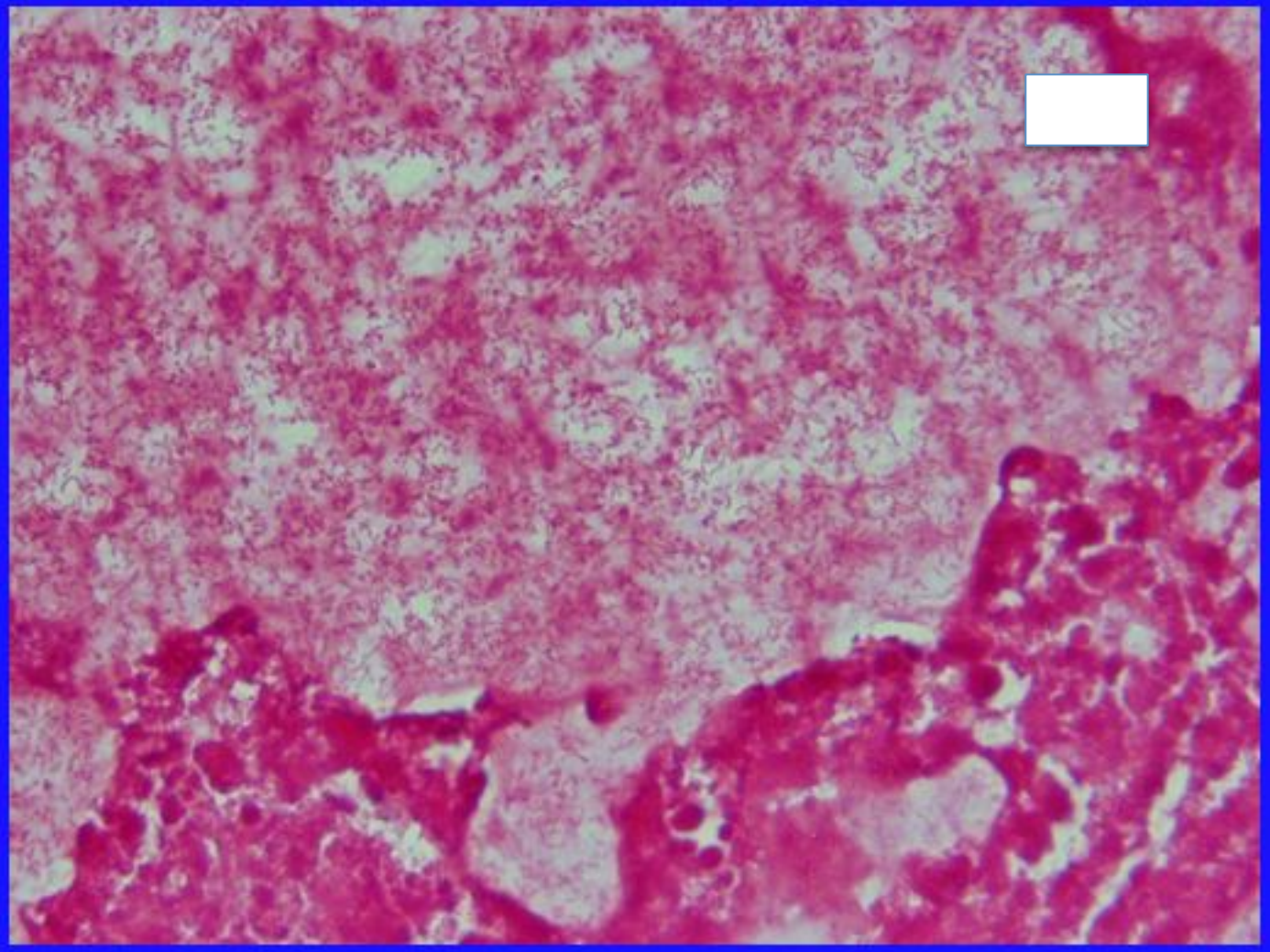


Mucoid biofilm of P. aeruginosa in an alveole surrounded by severely inflammed tissue (PMNs, pneumonia)

Strong immune response in the respiratory zone

Autopsy (BS242/74) of a Danish CF girl (MLM) who died due to chronic P. aeruginosa lung infection and 21 precipitating antibodies against P. aeruginosa. HE stain x 1000

HØIBY 2004



CF male, 41 years, chronic P.a. M+NM, 28 years, 46 precipitating antibodies, 114 2-week anti-P.a. treatment courses, explanted lung, Gram stain, x 1000 Høiby 2006

Serum antibody response of CF patients to P. aeruginosa alginate (Median (10-90 percentiles), Elisa Units)

Antibody class	CF - P. aeruginosa	CF + intermit. P. aeruginosa colonization	CF + chronic P. aeruginosa infection
Anti-alginate IgA	8 (0-40)	17 (2-98)	108 (29-240)
Anti-alginate IgG	8 (0-48)	25 (5-105)	252 (68-585)

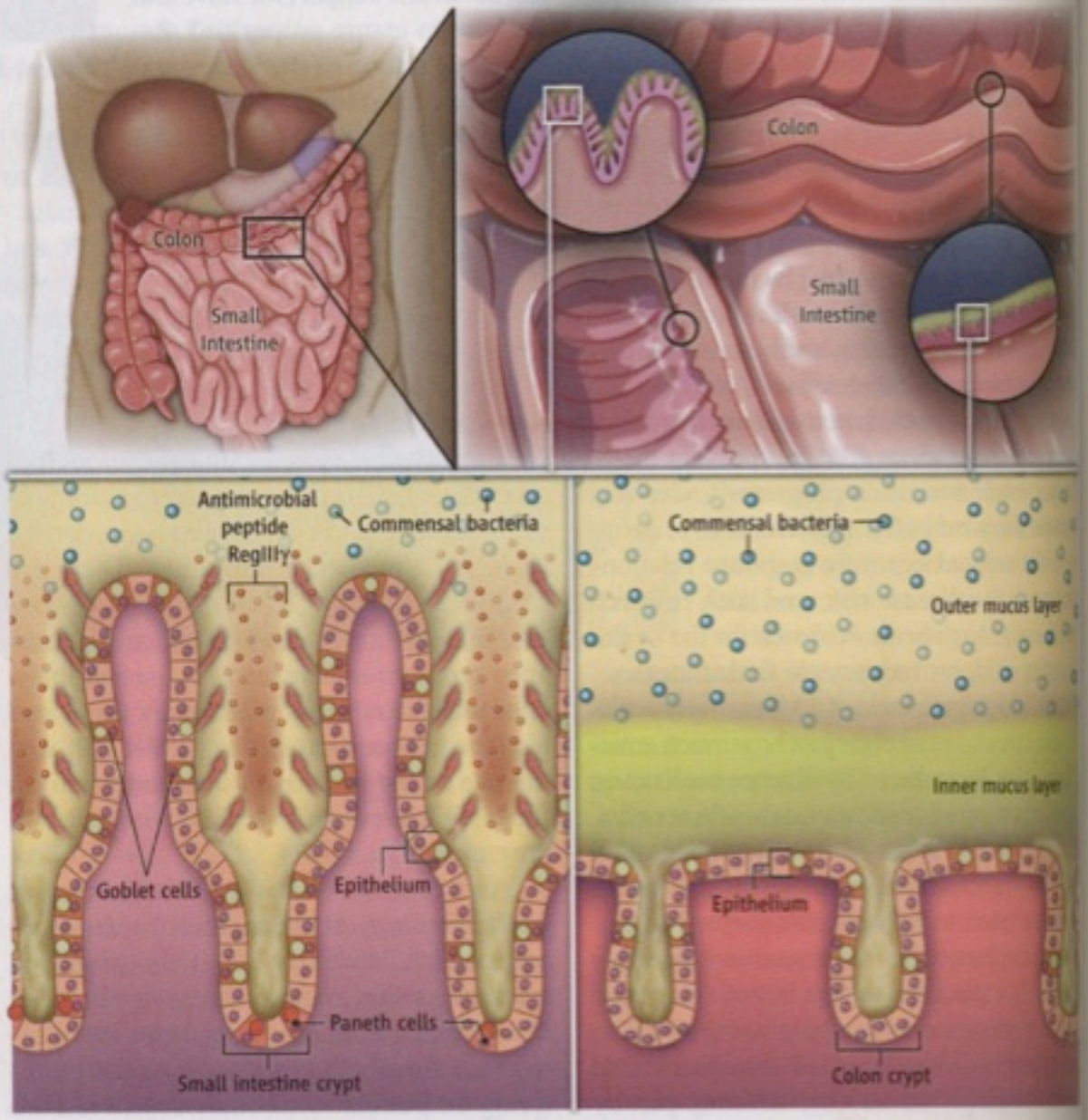
(Pedersen, Espersen, Høiby, Jensen: Immunoglobulin A and Immunoglobulin G antibody responses to alginates from P. aeruginosa in patients with cystic fibrosis. J. Clin. Microbiol. 28:747-755; 1990)

TNF-α concentration in sputum samples (pg/ml) from cystic fibrosis patients with chronic P. aeruginosa lung infection – Immune-complex mediated inflammation and tissue damage

	% positive*	mean (range)
Cystic fibrosis + LPS-immune complexes (N = 11)	82%	718 (0-1750)
Cystic fibrosis – LPS-immune complexes (N = 15)	27%	177 (0-1000)

*TNF-α > 30 pg/ml (ELISA method)

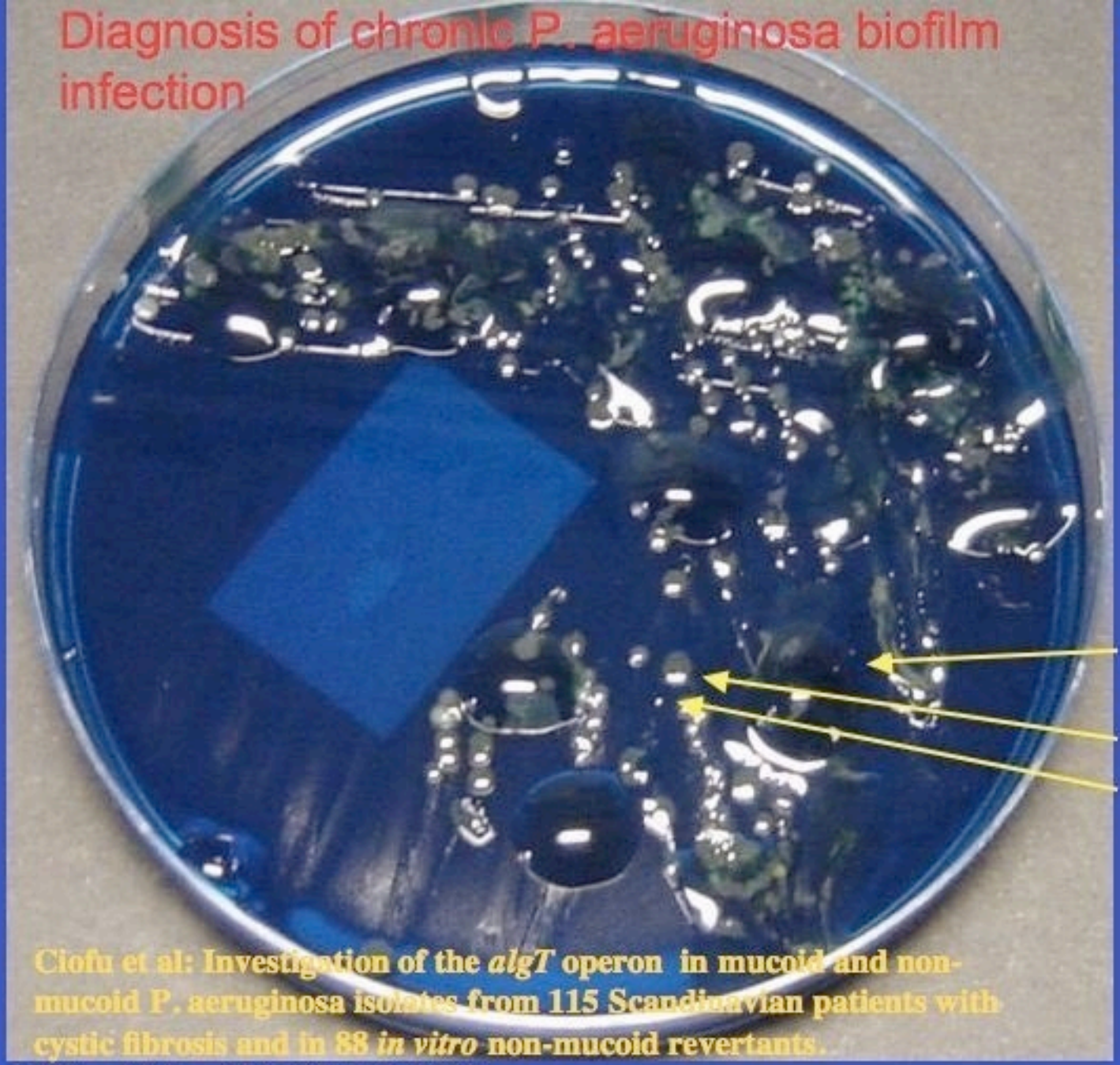
(Kronborg, Fomsgaard, Shand, Lanng, Hansen, Pressler, Høiby: TNF-alpha and Immune complexes in sputum and serum from patients with cystic fibrosis and chronic Pseudomonas aeruginosa lung infection. Immmunology and Infectious Dis.2 : (1992);171-177)



Intestinal protection. The intestinal epithelium is covered by mucus. In the colon, the inner stratified mucus layer acts as a physical barrier separating bacteria from the epithelium. The commensal bacteria are kept at a distance in the penetrable outer mucus layer. The small intestine is only covered with penetrable mucus where antibacterial molecules such as RegIIIγ generate a bacteria-depleted region close to the epithelium.

Johansson & Hansson: Keeping bacteria at a distance. Science 334:182-183; 2011

Diagnosis of chronic P. aeruginosa biofilm infection



CF FKM 070874 M Cph, Sputum 71467/02, 3 days at 37C and 1 day at 21C, Mucoid and non-mucoid and Small Colony Variants (SCV) P. aeruginosa

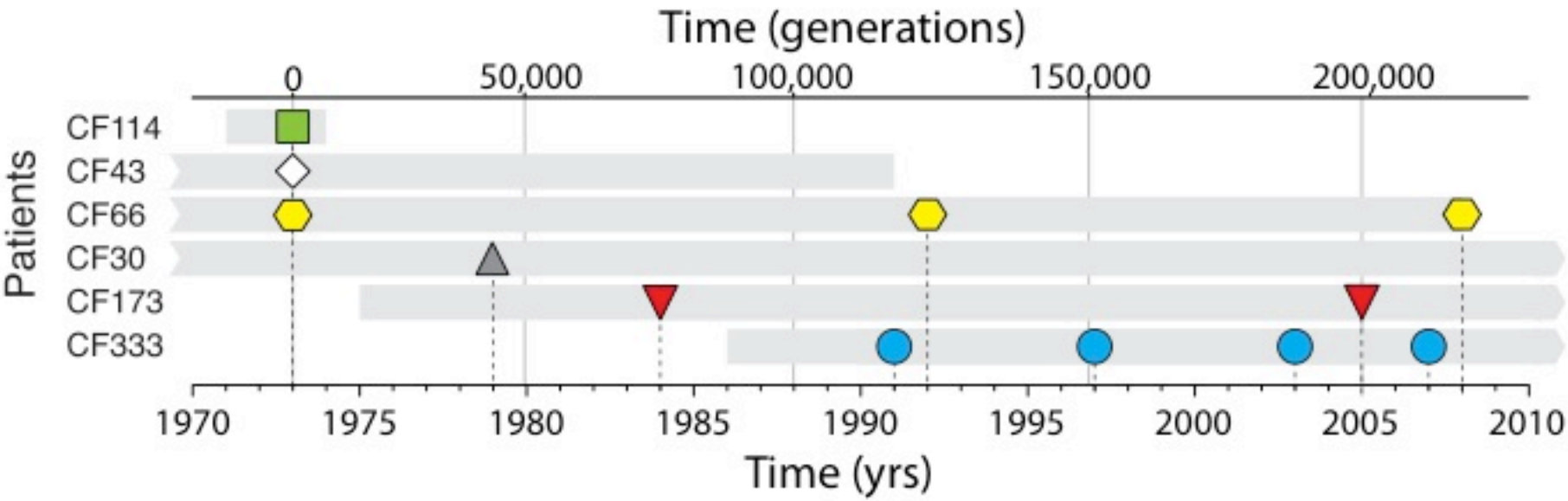
Ciofu et al: Investigation of the *algT* operon in mucoid and non-mucoid P. aeruginosa isolates from 115 Scandinavian patients with cystic fibrosis and in 88 *in vitro* non-mucoid revertants. Microbioloy,154:103-113; 2008.

Høiby 2009

Why M-NM-SCV phenotypes?

Adaptive divergens?

DR-2 clone

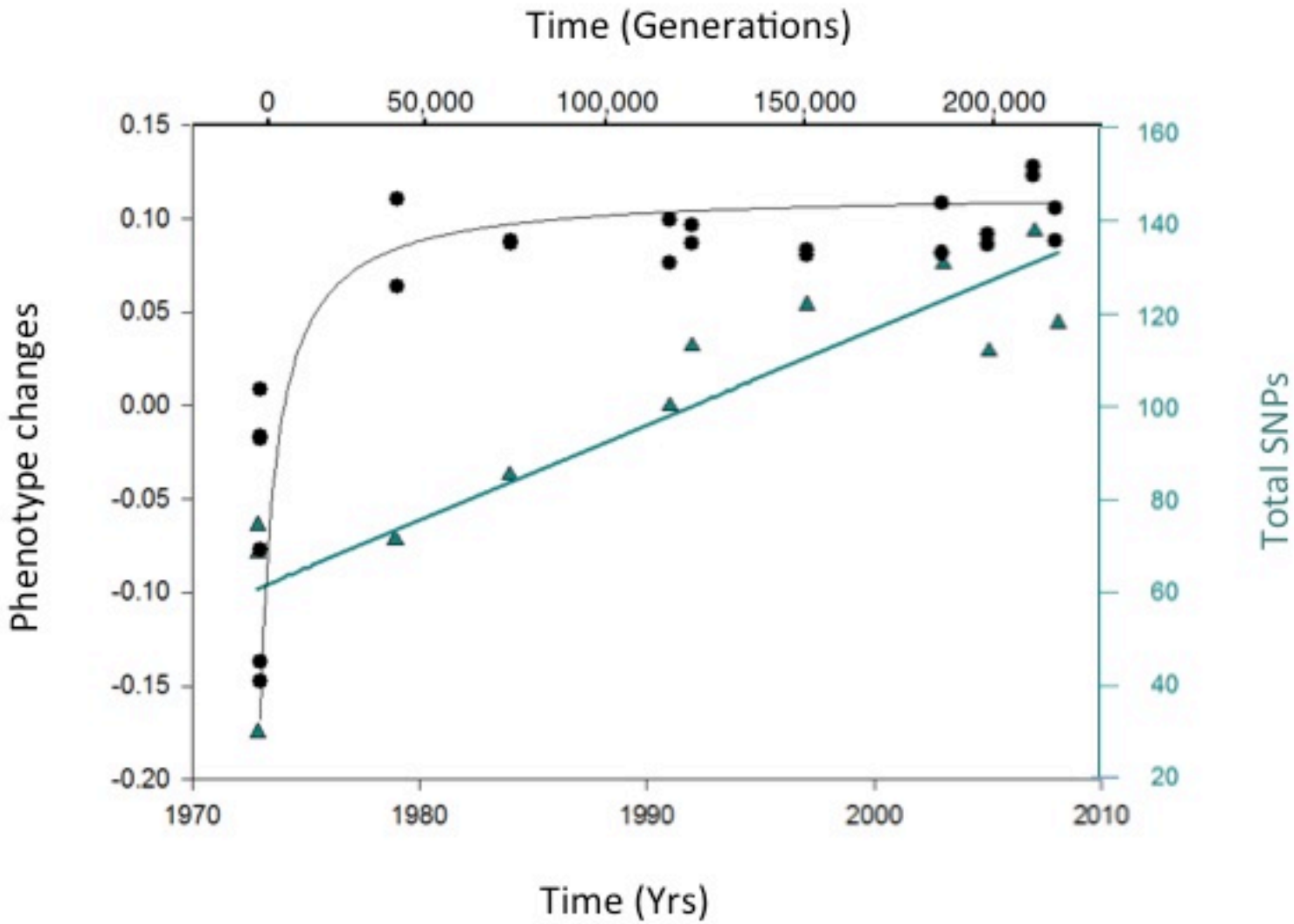


Isolate sampling points and patient life span. DK2 *P. aeruginosa* isolates were collected from six different CF patients during a 35 year time period. Bacterial isolates are represented by the colored symbols, and grey bars represent patient lifespan

(Yang, Jelsbak, Marvig, Damkier, Workman, Rau, Hansen, Folkesson, Johansen, Ciofu, Høiby, Sommer, Molin: Evolutionary dynamics of bacteria in a human host environment. PNAS 108:7481-7486; 2011)

Figure 4

DR-2 clone

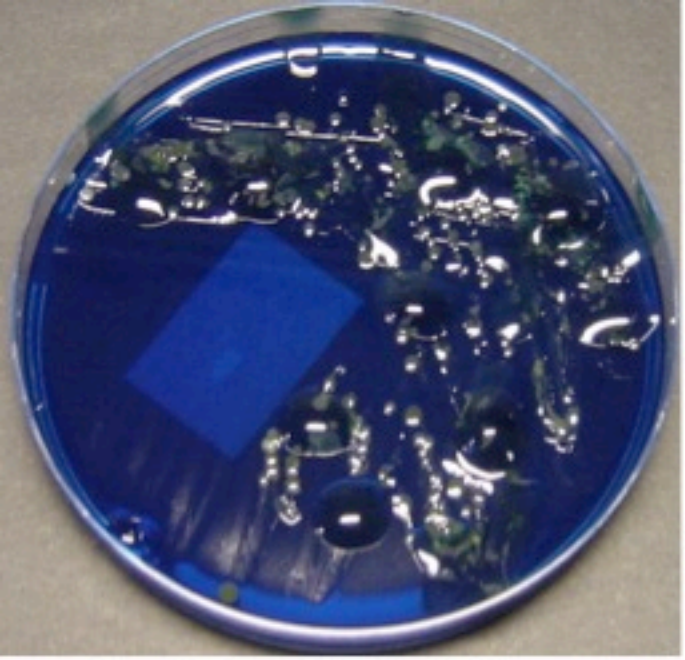


Folkesson et al. Nature Reviews, submitted 2011

Table S1: Selected phenotypes and gene sequences. DR-2 clone

Strain[a]	*in vitro* doubling time (min)[b]	Bacterial generation	*mucA*	*algU*	*rpoN*	*lasR*	3-O-C_{12}-HSL[c]
PAO1	26		wt	wt	wt	wt	+
CF114-1973	26	0	C382T, nonsense	C448G[d]	wt	wt	+
CF43-1973	34	0	C352T, nonsense	G349T, nonsense	wt	wt	+
CF66-1973	50	0	ΔG430	A55G; 116-117insTGCG	T1256C	G647A[e]	+
CF30-1979	51	42616	ΔG430	A55G	T1256C	deletion	-
CF173-1984	63	74279	ΔG430	A55G	T1256C	deletion	-
CF173-2005	72	195178	ΔG430	A55G	T1256C	deletion	-
CF333-1991	56	118607	ΔG430	A55G	T1256C	deletion	-
CF333-1997	59	156602	ΔG430	A55G	T1256C	deletion	-
CF333-2003	74	185534	ΔG430	A55G	T1256C	deletion	-
CF333-2007	78	204822	ΔG430	A55G	T1256C	deletion	-
CF66-1992	62	124939	ΔG430	A55G	T1256C	deletion	-
CF66-2008	64	209644	ΔG430	A55G	T1256C	deletion	-

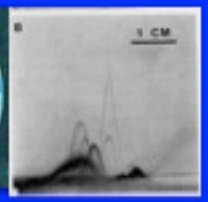
(Yang, Jelsbak, Marvig, Damkier, Workman, Rau, Hansen, Folkesson, Johansen, Ciofu, Høiby, Sommer, Molin: Evolutionary dynamics of bacteria in a human host environment. PNAS 108:7481-7486; 2011)



Evolution of *P. aeruginosa by* adaptation to the chronic stressfull lifestyle in The 3 Niches of the United Airways of Cystic Fibrosis Patients by means of continuous mutations and selection of THE FITTEST CHRONIC PHENOTYPES

- Adaptation to the non-inflammatory (s-IgA) and inflammatory defense system (IgG, PMNs
- Adaptation to the microaerophilic/anaerobic paranasal sinuses
- Adaptation to the anaerobic sputum in the conductive zone of the lungs and in abscess-like areas of infection
- Adaptation to the aerobic respiratory zone of the lungs: alveoles, respiratory bronchioles
- Adaptation to the antibiotic therapy
- PMN-ROS contribute to the mutation rates leading to strong and weak mutators and subsequently multiply antibiotic resistance
- The global regulators *mucA, AlgT, RpoN and lasR* are the most important genes for the chronic phenotypes
- The 2 major adaptive mechanisms are alginate production and biofilm formation
- The tissue damage is caused by chronic immune-complex mediated PMN inflammation around the persisting biofilms

EUREKA - THE STANDARD MODEL OF P. AERUGINOSA INFECTION IN CYSTIC FIBROSIS



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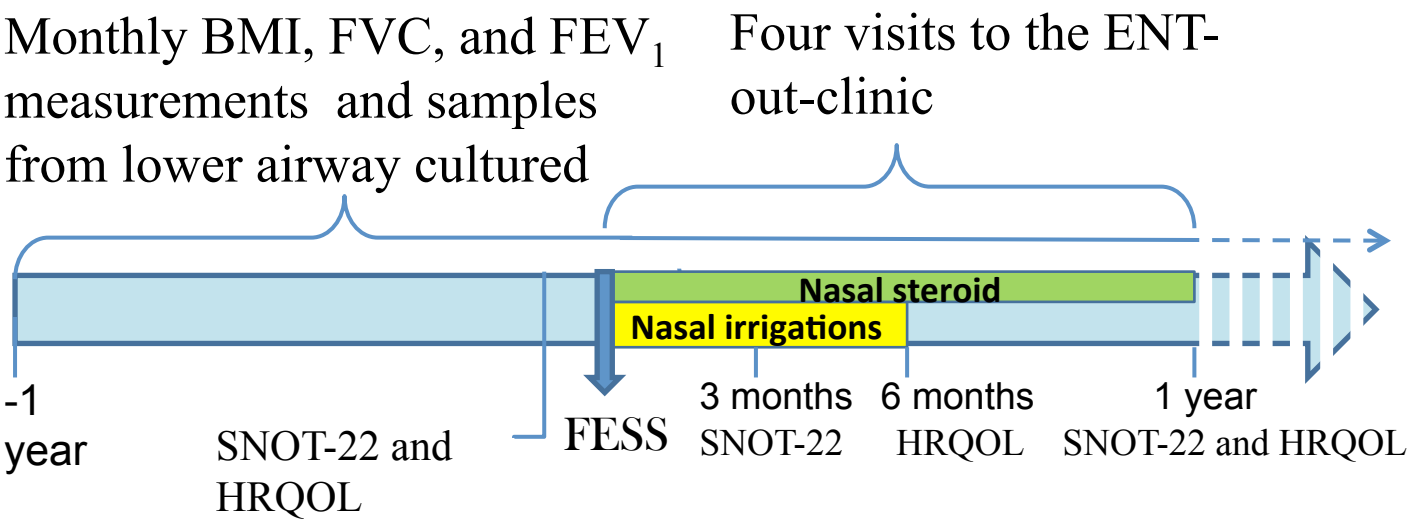

(Aasnæs, Johansen, Skov, Buchvald, Hjuler, Pressler, Høiby, Nielsen, Buchwald: Clinical effects of sinus surgery and adjuvant therapy in cystic fibrosis patients – can chronic lung infections be postponed? J.Cystic Fibrosis, 2012, submitted)

(Aasnæs, Johansen, Skov, Buchvald, Hjuler, Pressler, Høiby, Nielsen, Buchwald: Clinical effects of sinus surgery and adjuvant therapy in cystic fibrosis patients – can chronic lung infections be postponed? J.Cystic Fibrosis, 2012, submitted)

Lung infection status at FESS	Lung infection status a year after FESS	
CF-: 16 patients	CF-:	14 patients
	CF+(i):	2 patients
	CF+(c):	0 patients
CF+(i): 61patients	CF-:	22 patients
	CF+(i):	35 patients
	CF+(c):	4 patients
CF+(c): 29 patients	CF-:	4 patients
	CF+(i):	1 patients
	CF+(c):	24 patients

The one-year prevalence of intermittent colonization CF+(i) **decreased** by 38% (CI: 24%–51%) after FESS (58% at surgery compared with 36% one year postoperatively; $p<0.01$).
The one-year prevalence of non-colonized patients (CF-) **increased** by 150% (CI: 71%–310%) after FESS (15% at surgery compared with 38% one year postoperatively; $p<0.01$).